Proceedings
of the
Society
for
Experimental Biology and Medicine

Vol. 77 July, 1951 No. 3

SECTION MEETINGS

BALTIMORE	
Camp Detrick, Md.	June 27, 1951
MISSOURI	
Washington University	May 16, 1951
SOUTHERN CALIFORNIA	
University of Southern California	June 21, 1951

Effect of Prolonged High Sodium Chloride Ingestion and Withdrawal upon Blood Pressure of Dogs.* (18784)

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The effect of salt ingestion upon blood pressure of dogs with renal hypertension or with partial renal insufficiency has been studied (1,2) but there seems to be a paucity of information on the effect of excess salt ingestion on normal dogs.

Methods. Three well trained, standardized dogs weighing 14.1, 16.3, and 15.3 kg were used. During the control period which lasted for approximately 2 months, the animals were fed the kennel diet of Nutrena dog food. Blood pressure was determined by the auscultatory method of Allen(3) with certain modifications introduced by the authors(4). During the control and experimental period, blood pressure was determined 6 days each week. The daily values as recorded in Table I are the means of at least 10 consecutive readings. In the *first phase* of excess salt ingestion, salt was mixed with the kennel diet and the animals were allowed water *ad libitum*. In Dogs I, II and III, the amount given was 30, 40 and 40 g daily. These amounts represented approximately 2.1; 2.5 and 2.6 g per kg, respectively. Dog I was kept on this regimen for 205 days; Dog II for 56 days, and Dog III for 154 days. In the *second phase* of excess salt ingestion which followed the first phase without interruption, the animals were given a 2% sodium chloride solution as the sole source of drinking water. This period lasted 49, 49 and 35 days in the 3 dogs and was terminated because of the advent of warm weather and evidences of toxic effects. The total time of excess salt ingestion (Phase I plus Phase II) was 254, 105 and 189 days.

Results. Phase I. The results and the

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* Financed in part by a grant from the Nebraska Medical Foundation.

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TABLE I. Blood Pressure of Dogs on Excess Salt Intake.

Dog	Conditions	Mean	S. D.*	C. V.†	C. R. with control‡	P§	N‖
I	Control	95/50	7.2	7.6	—	—	32
			6.5	13.1	—	—	
	20 g NaCl	107/51	9.7	9	4.6	>.01	20
	Struggle		6.8	13.3	.5	<.50	
	30 g NaCl	115/59	12.4	10.8	6	>.01	16
	Struggle		7	11.9	4.3	>.01	
	30 g NaCl	107/54	8.7	8.1	2.9	>.01	11
	Basal		5.6	10.7	1.92	<.05	
	2% NaCl	104/53	14.7	14.1	3.4	>.01	40
	Drink		9.1	17.2	1.6	<.1	
	After NaCl	78/37	6.4	8.2	8.5	>.01	18
	Stopped		6.8	18.4	6.6	>.01	
II	Control	98/55	5.5	5.6	—	—	44
			7.8	14.2	—	—	
	30 and 40 g NaCl	117/57	9.8	8.4	6.9	>.01	14
	Struggle		10.2	17.8	.6	<.50	
	40 g NaCl	97/51	9	9.2	.7	<.5	24
	Basal		6	11.8	2.4	>.02	
	2% NaCl	103/49	13.5	13.1	2.16	>.01	39
	Drink		10	20.3	3	>.01	
	After NaCl	84/41	6.6	7.9	8	>.01	18
	Stopped		4.8	11.6	8.6	>.01	
III	Control	98/50	5.7	5.8	—	—	40
			6.3	12.5	—	—	
	30 and 40 g NaCl	118/50	21.1	17.9	3.74	>.01	10
	Struggle		5.1	10.3	0	—	
	40 g NaCl	101/45	6	6	1.37	<.10	9
	Basal		6.9	15.2	2	<.05	
	2% NaCl	98/43	21.2	21.7	0	—	28
	Drink		7.9	18.4	3.9	>.01	
	After NaCl	82/41	5.8	7.1	9.1	>.01	15
	Stopped		7	17	4.4	>.01	

* Std. dev. of mean.

† Coefficient of variation.

‡ Critical ratio of mean experimental systolic and diastolic pressure compared with control.

§ Probability that observed changes in the mean are not due to chance.

‖ No. of daily blood pressure determinations in each period.

statistical analyses are shown in Table I. Any change in the mean values which was equal to or greater than the 5% level of confidence ($P \geqq .05$) was considered significant. Each of the 3 dogs showed a similar pattern of reaction which could be divided into 2 periods as follows: (A) *The period of struggle.* Immediately after starting the excess salt ingestion the blood pressure began to show markedly increased daily variations, which were more pronounced in the systolic than in the diastolic pressure. The daily weight also began to show wide fluctuations sometimes as much as 1.5 kg in 24 hours, but the mean weight for any period was usually insignificantly different from the control weight. During this period of struggle, the mean systolic pressure was significantly elevated above the control value in all dogs. In only one dog, however, (Dog I on 30 g) was the diastolic pressure significantly elevated. The duration of the period of struggle was variable, lasting from 9 to 21 days. (B) *The salt basal period or period of compensation.* Following the period of struggle the pressure began to show smaller daily fluctuations, and the previously elevated systolic pressure declined. These changes were interpreted as an adaptation or compensation to excess salt intake. As soon as adaptation occurred the administration of excess salt was continued and the animals were subjected to a second type of stress, namely, confinement in a cage treadmill, which revolved between one and two miles per hour. They were kept in this cage for many periods ranging from 3 to 18 hours daily. The details of these experiments are not included in this paper. It will suffice to say that the initial effect of the second stress was a great increase in blood pressure. For example, following two consecutive nights in the revolving cage treadmill the blood pressure of Dog III rose to 215/80 and it required 10 days to return to the previous level, when this stress was discontinued. Continuation of the second stress, however, resulted in adaptation and the increase in blood pressure became small or insignificant.

Phase II (2% NaCl as drinking water). The salt water was well tolerated and the intake, which varied with the temperature, ranged from 500 to 3000 cc in 24 hours. The effect upon blood pressure was surprisingly small. In all 3 dogs, it differed but slightly from that during the salt basal or compensatory period. The daily fluctuations in pressure were greater than in the period of struggle of Phase I as shown by the increased coefficients of variation. Body weight fluctuated markedly but the mean changes for the period were small. The results obtained in Phases I and II were clearly indicative of a progressively increasing adaptation to the ingestion of excess salt.

Phase III (After discontinuation of excess salt intake). When excess salt administration was suddenly stopped, all 3 dogs immediately showed a marked and highly significant decrease in both systolic and diastolic pressure below the control level which persisted for at least 3 weeks. The coefficients of variation for the systolic pressure decreased and approximated the control values.

A fourth dog was recently given 40 g of sodium chloride daily with the food for 18 days and then given 2% sodium chloride solution to drink for 57 days (a total of 75 days on excess salt). At the end of 57 days of drinking 2% saline the animal became violently ill and the experiment was terminated. The blood pressure of this dog showed less change during the period of excess salt ingestion than was observed on the other 3, but when excess salt ingestion was stopped the blood pressure failed to drop below the control value. Either the period of excess salt ingestion was too short or there are individual differences in the ease with which this phenomenon can be induced.

Discussion. It is obvious that sustained hypertension cannot be produced in normal dogs by the ingestion of excess salt, probably because the dog kidney has phenomenal ability to eliminate excess salt(5). The same observation has been made by Allen(13). The increases in the coefficients of variation suggest that fluid and mineral equilibrium was maintained by virtue of an intense struggle. Eventually the adaptive mechanisms became more efficient and the salt basal or compensation pressures were established, fluctuations in pressure, as indicated by the coefficients of variation then approached the control level. Saperstein *et al.*(6) have pointed out that when excess salt is ingested, but the animal allowed water *ad libitum,* the fluid intake may become so high that the animal is able to excrete the excess salt. They state that fluid intake should be limited when excess salt is given and suggest that this be done by giving 2% saline solution as the sole source of drinking water. In rats this causes definite hypertension(6,7). In the present studies, Dogs I, II, and III had been on a high salt intake for 205, 56 and 154 days before 2% saline ingestion was started and the adaptive mechanisms had become so perfected that elevation of blood pressure above the salt basal or compensatory level was entirely prevented in Dogs I and III. In Dog II there was a significant elevation of systolic pressure above the salt basal value ($P > .05$). This may be due to the fact that this dog had been on excess salt ingestion for a much shorter period than the other 2 dogs, hence the adaptive mechanisms were not as highly developed.

The marked decrease in blood pressure following the sudden withdrawal of salt was an unexpected finding, the exact cause is unknown but we suggest the following hypothe-

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sis: During the prolonged period of high sodium ingestion, the secretion of mineralo-corticoids from the adrenal cortex dropped to low levels, since there was no need for conservation of sodium. When excess sodium administration was suddenly stopped, the familiar phenomenon of glandular inertia occurred and mineralo-corticoid secretion remained low (in these experiments for at least 3 weeks). This hypothesis necessitates two other implications, (1) that mineralo-corticoids are normally responsible for maintaining blood pressure at a higher level than it would be in their absence and (2) that excess salt ingestion *per se*, was responsible for maintaining the blood pressure above the post withdrawal level. It is important to emphasize that during the period of low blood pressure, the animals showed no gross evidence of adrenal insufficiency. The above hypothesis is supported by direct evidence obtained by cytological studies of the adrenal cortex. Deane, Shaw, and Greep(10), Selye and Stone(11), and Bacchus(12) have shown that when the sodium-potassium ratio is raised there is evidence of decreased activity in the glomerular zone. The administration of desoxycorticosterone produces similar changes. Lowering of the sodium-potassium ratio brings about changes indicative of increased activity in the glomerular zone.

It may be significant that the blood pressure level after sudden salt withdrawal was of the same order of magnitude as the stable fasting level which we have found in dogs(4). Selye (8,9) has postulated that the amount of adaptation energy is limited so that when an animal has become resistant to one type of stress, it is highly susceptible to a second stress. In the present experiments prolonged confinement (up to 18 hours per day) in a slowly revolving cage treadmill was used as a second stress. The first effect was a marked and sometimes prolonged elevation of systolic and diastolic pressure, but as the second stress was continued, the response became progressively less, hence adaptation to the second stress was marked even when the first stress was continued. It is possible that the second stress was not of sufficient intensity to elicit the effect described by Selye. It has not been possible to standardize the response to the revolving cage treadmill with sufficient accuracy to state whether dogs on high salt intake are more sensitive to this type of stress than normal dogs.

Summary. 1. Three well trained standardized dogs were given excess sodium chloride in the diet in amounts approximating 2.5 g per kg weight for 205, 56 and 154 days respectively. The first effect was a marked increase in the daily variations in blood pressure and a small but significant elevation of the systolic pressure. In only one dog was the diastolic pressure elevated. As excess salt ingestion was continued there was evidence of increasing adaptation characterized by a drop in the previously elevated systolic pressure. Adaptation finally became so highly developed that when the salt stress was increased by giving 2% saline solution as the sole source of drinking water, 2 dogs showed no additional elevation in systolic pressure while one showed a small but significant elevation. 2. After adaptation to salt was complete, a second stress caused elevation of both systolic and diastolic pressure but continuation of the second stress led to adaptation. 3. The sudden withdrawal of excess salt after administration for 254, 105 and 189 days, respectively, was quickly followed by a marked and highly significant fall in both systolic and diastolic blood pressure, which persisted for at least 3 weeks. A possible cause for this drop in pressure is discussed.

Received April 30, 1951. P.S.E.B.M., 1951, v77.

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Effect of Excluding Pancreatic Juice from Duodenum on Secretory Response of Pancreas to a Meal. (18785)

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In experiments in which all pancreatic juice is collected and thereby prevented from reaching its physiologic destination, an abnormal situation is created which could alter the natural pancreatic secretory process. We have carried out the following experiments to ascertain whether the postprandial secretory activity of the pancreas is modified when the juice is excluded from the duodenum.

Methods. Pancreatic juice was obtained from 4 trained dogs by Scott and associates' (1) modification of the method of Thomas and Crider(2). A metal cannula was installed in the duodenum directly opposite the opening of the major pancreatic duct. At the time of this surgical procedure, the minor pancreatic duct was ligated and divided. During experiments the major pancreatic duct was cannulated, via the larger metal cannula, with a fine glass cannula. Between experiments the glass cannula was removed, the metal cannula was capped, and pancreatic juice flowed into the duodenum in a normal manner. Pancreatic secretion was stimulated by the feeding of 200 g of lightly cooked horse meat. Juice was collected for 3 hours after feeding. Four series of experiments were performed. In the first, all pancreatic juice was collected and withheld from the animals. In the second, pancreatic juice was collected, its volume was measured, and it was placed in the duodenum at intervals of 5 minutes. Thus, the animals retained the pancreatic juice they secreted, though they received it in bursts and from 0 to 5 minutes later than would have been the case under normal circumstances. In the third, all juice was collected and withheld, but equivalent volumes of isotonic saline solution were placed into the duodenum, at intervals of 5 minutes. In the fourth, all juice was collected and withheld, and a tenth normal solution of sodium bicarbonate was continuously instilled into the duodenum through the Thomas cannula during the experiment by means of a continuous drip apparatus of the type commonly used for intravenous fluid therapy. Fifteen centimeters of sodium bicarbonate were instilled during the 15 minute period preceding feeding, 120 cc per hour for the first 1½ hours after feeding and 40 to 60 cc per hour for the second 1½ hours after feeding. Thus the quantity of bicarbonate solution reaching the duodenum was from 2 to 4 or more times the quantity reaching it in the form of pancreatic juice during normal digestion. The volume of juice secreted was measured in all instances. Except when all juice was instilled into the duodenum, measurements were also made of alkali content by the method of Van Slyke(3), and of total nitrogen content by the micro-Kjeldahl technic.

Results. Data regarding the volume, bicarbonate content and total nitrogen in pancreatic juice secreted during the first 3 hours after feeding are presented in Table I. Considerable variation occurred in the results obtained. When all juice was replaced into the duodenum, the volume of juice secreted by the 4 dogs in 3 hours averaged 40.7, 42.1, 55.2, and 73% of comparable figures obtained when all juice was removed. Only in dog 3 did the ranges of determinations overlap. We interpret the differences as being significant. Because all juice was replaced in this series of experiments, no values for bicarbonate or nitrogen content were obtained.

When isotonic saline solution was substituted for pancreatic juice in the duodenum, volumes secreted during the 3 hour period averaged 75, 92.8, 74.1, and 113.1% of the mean values obtained when all juice was re-

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TABLE I. Total Volume, Bicarbonate and Nitrogen in Canine Pancreatic Juice Secreted During 3 Hr After Ingestion of a Meal of Meat.

			Vol., ml			Bicarbonate content, mEq			N content, mg		
Exp.	Dog	No. of exp.	Mean	Range	%*	Mean	Range	%*	Mean	Range	%*
All juice removed	1	5	51.9	41.6– 64	100	6.5	4.9– 8.6	100	126	111–139	100
	2	4	76	63.8– 85.1	100	10.5	8.4–12.1	100	200	180–236	100
	3	7	148.5	91.7–176	100	21.9	13.1–26.9	100	206	156–256	100
	4	4	97.9	81.3–116.2	100	13.1	10.8–15.9	100	185	150–218	100
All juice replaced	1	4	21.1	12.2– 26.5	40.7	—	—	—	—	—	—
	2	3	32	27.3– 38	42.1	—	—	—	—	—	—
	3	7	81.9	45.2–112.4	55.2	—	—	—	—	—	—
	4	4	71.5	56.4– 81.1	73	—	—	—	—	—	—
Juice replaced by isotonic saline sol.	1	3	38.9	30.5– 52.1	75	5	3.7– 7.4	76.9	124	108–140	98.4
	2	3	70.5	65.6– 72.9	92.8	9.8	9.4–10.1	93.3	196	139–240	98
	3	4	110.1	80.6–130.7	74.1	15.8	11.3–19.3	72.1	190	171–212	92.2
	4	3	110.7	87.7–144.9	113.1	15.8	11.8–22.1	120.6	176	148–216	95.1
HCO_3 sol. instilled into duodenum	1	3	21	15 – 27.9	40.5	2.3	2.1– 2.4	35.4	93	65–136	73.8
	2	3	27.2	23.5– 32.3	35.8	3	2.7– 3.6	28.6	127	104–157	63.5
	3	3	84.1	73 – 94	56.6	11	9.1–12.8	50.2	175	152–202	85
	4	2	51	38.9– 63.2	52.1	6.4	4.2– 8.6	48.9	149	122–176	80.5

* Mean values expressed as % of comparable values obtained when all juice was removed.

moved and nothing was replaced into the duodenum. Ranges overlapped in all 4 dogs and we do not consider the differences to be significant. Likewise, replacement of juice by isotonic saline solution did not significantly alter the output of bicarbonate or nitrogen from that which occurred when nothing was substituted for the pancreatic juice.

Introduction of the stated quantities of sodium bicarbonate solution into the duodenum, however, reduced the mean volume of pancreatic juice secreted to 40.5, 35.8, 56.6, and 52.1% of values observed when no replacement was made, values essentially similar to those obtained when juice was returned to the duodenum. Again, ranges of determinations overlapped only in dog 3, in which the secretory response seemed more variable than in the other dogs. The total bicarbonate secreted was also markedly less when sodium bicarbonate was instilled into the duodenum. The total nitrogen, though less on the average than that observed when no bicarbonate solution was placed in the duodenum, again showed overlapping of the ranges of values in 3 of the 4 dogs and the difference is of doubtful significance.

When data for successive samples collected during periods of 15 minutes were plotted against time, it was noted that when pancreatic juice was replaced into the duodenum the general shape of the secretory curve remained unchanged though it was quantitatively depressed.

Comment. Of the 4 series of experiments in the present study, that in which pancreatic juice was returned to the duodenum most closely approximates the normal state. A similar quantity of juice was secreted when large amounts of sodium bicarbonate were substituted for pancreatic juice. A larger quantity of juice was secreted when pancreatic juice was excluded from the duodenum both with and without the intraduodenal instillation of a nonalkaline solution (isotonic saline solution). These events can be explained by assuming that the alkalinizing effect of pancreatic juice normally secreted in response to a meal is sufficient to minimize the number of instances in which the pH of duodenal contents becomes low enough to activate the secretin mechanism, and that this alkalinizing effect does not occur when all pancreatic juice is excluded from the duodenum. If the acid-secretin mechanism played a dominant role in stimulation of postprandial pancreatic secretion one would expect instillation of an excess of alkali into the duodenum to result in a still lower volume of juice than that secreted when normally secreted pancreatic juice alone was replaced. Since this was not the case, the data suggest that the acid-secretin mechanism does not play a major role in the response of the canine pancreas to a meal of meat under normal circumstances. Studies of the importance of products of digestion of protein and fat as pancreatic secretagogues and the discovery of pancreozymin have demonstrated other physiologic mechanisms capable of stimulating the pancreas during normal digestion. The protective role of the acid-secretin mechanism in instances of gastric hypersecretion seems unquestionable. Data concerning this concept have been reviewed recently by Thomas(4).

Conclusions. 1. When canine pancreatic juice secreted in response to a meal was permanently removed by the method used in these experiments, the total volume of juice secreted was significantly greater than was the case when the juice was replaced in the duodenum. 2. A similar reduction in the volume of postprandial pancreatic secretion occurred when tenth normal sodium bicarbonate solution was instilled into the duodenum in place of pancreatic juice. 3. No similar reduction in secretion occurred when equivalent volumes of isotonic saline solution were substituted for pancreatic juice.

Received May 2, 1951. P.S.E.B.M., 1951, v77.

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Probable Mechanism of Alizarin Inhibition of Calcification. (18786)

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Alizarin red S has been found to prevent calcification in embryonic metatarsals without interference with normal cartilage and fibrous connective tissue growth(1). This paper describes our attempts to determine the mechanism of the inhibition by observing the effects of alizarin on the enzyme, phosphatase, as well as on the calcium and phosphate ions.

Experimental. Alizarin and bone phosphatase. Because of the diversity of materials and methods employed, these will be presented along with our experimental results. The effect of alizarin on phosphatase production by many paired metatarsals and fibulas from 10-day-old chick embryos was studied first. One member of each pair was placed on the surface of a hanging drop consisting of equal parts of blood plasma and embryonic extract; the other member was treated similarly save that the medium contained one drop of a 1% Seitz-filtered aqueous alizarin solution to 120 drops of medium. At this dye concentration, calcification is inhibited. After 4 days of cultivation both members of live and fixed (95% alcohol) preparations were placed either in calcium nitrate as controls or in glycerophosphate substrates for phosphatase visualization. The preparations were then treated with silver nitrate, washed with distilled water and mounted in glycerine gelatin. Phosphatase was found abundantly distributed in the paired bones from both the alizarin-containing and alizarin-free media. Thus, no indication of bone phosphatase inhibition by alizarin in cultures was observed. The *in vitro* effect of alizarin on phosphatase was examined next. To chick alkaline bone phosphatase(2), assaying 46.8 Bodansky units per 100 ml(3), alizarin red S was added in an amount (0.6 mg per ml) approximately 6 times that required to inhibit calcification in tissue culture. There was no significant reduction in phosphatase activity (45.2 Bodansky units per 100 ml). Since bone phosphatase is not inactivated by alizarin either

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in tissue culture or in the test tube, some other locus of dye action was sought.

Alizarin and calcium. Since alizarin can form a lake with calcium, the question arose whether the calcification inhibition observed was due to disionization of calcium in the medium. The clotting time of the blood media with and without the presence of dye in calcification-inhibiting concentration was recorded to determine whether this amount of alizarin was removing calcium and hence delaying clotting. The first appearance of fibrin threads on the cataract knife used for mixing was recorded as the clotting time. No difference in clotting times was observed. However, if the dye concentration was increased 5 to 10 times the minimal calcification-inhibiting concentration, clotting was distinctly retarded. Further evidence that alizarin did not disionize the calcium in the medium in order to prevent calcification was obtained by comparing the behavior of 50-hour embryonic chick hearts grown for 2 days on alizarin-containing and dye-free media. New growth appeared from the cut ends of each type of preparation and the average rates of beat of the two types were practically identical (94 times per min. on alizarin; 114 times per min. on alizarin-free media). Since activity of this kind cannot occur unless the calcium ion is present(4), the conclusion is obvious.

TABLE I. Effect of Alizarin and Litmus on Paired Embryonic Chick Fibulas Grown in Media Containing the Respective Dyes.

	Alizarin		Litmus	
Pair of fibulas	Total length, mm	Mineralization zone, mm	Total length, mm	Mineralization zone, mm
1	4	.50	4.20	1
2	4	.55	4.25	.75
3	4.05	1.10	4	1.15
4	4	.90	4.10	1.10
5	4.5	.50	4.20	1.20
6	4.40	1.05	4.35	1.25
7	3.60	.50	3.60	.75
Avg	4.08	.73	4.10	1.03

Litmus and calcification. Since litmus, like alizarin, is a dye having an affinity for calcium(5), we compared the two for effect on clotting time and calcification. A purified litmus was prepared from the crude dye(6), and a 1% litmus-Tyrode solution made. For both the clotting and calcification experiments, the clots were composed of equal parts of blood plasma and a mixture consisting of Tyrode solution, embryonic extract and the respective dye. The litmus concentration used was approximately 15 times greater than that of alizarin since it was found from preliminary experiments that this concentration of litmus had an effect on clotting time but not on calcification. The average clotting time of 10 litmus drops was 4 min. while that of an equal number of alizarin drops was 2.5 min. Apparently litmus, unlike alizarin, removed sufficient calcium ions from the medium to increase the clotting time. For a comparison of calcification in litmus and alizarin, the same media described for the clotting experiments were used. One member of each pair of a series of femurs, tibias, fibulas and metatarsals from 9-day-old chick embryos was placed on an alizarin clot and the other on a litmus clot and grown for 3 days. After this period, 2 drops of alizarin-Tyrode solution (1 drop of a 1% aqueous alizarin solution to 59 drops of Tyrode solution) were added to each culture, litmus as well as alizarin, in order to visualize the extent of mineralization. After 5 hours, measurements of total lengths and of the lengths of the mineralization zones were made for each bone. Typical data for one such experiment on 7 pairs of fibulas are presented in Table I. It can be seen that average total lengths of the paired fibulas are practically the same when grown in the presence of either dye. As regards the zones of mineralization, however, the fibulas grown on litmus show about 40% more calcification than do those grown on alizarin (1.03 mm as against 0.73 mm). Unlike alizarin, then, litmus will not stop calcification in concentrations which interfere with the clotting process. It appears that, in the medium in general, a higher concentration of calcium ions is necessary to promote plasma clotting than is re-

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quired for calcification.

Alizarin and the medium. To determine whether alizarin inhibits calcification by rendering some constituent of the medium unavailable to the growing bone, bones grown on alizarin medium were transferred to dye-free medium. As a precautionary measure, the bones were washed and placed side by side with the controls in dye-free medium. Over a period of 4 days, calcification was not resumed in the alizarin-stained bone although the cartilage and fibrous connective tissue grew at a normal rate. In contrast, the control bone of each pair always continued to show calcification as well as normal growth of cartilage and connective tissue.

Discussion. These observations demonstrate that calcification can be stopped in tissue culture without inhibiting phosphatase and without removing the calcium and phosphate ions to a significant degree from the medium in general. The effect of alizarin, therefore, must be on some other mechanism or at some specific locus within the hanging drop culture. We are inclined toward the latter supposition and will presently develop it more fully.

Throughout the hanging drop medium there is a uniform and abundant distribution of phosphatase as well as calcium and phosphate compounds. Under these conditions the concept of the operation of a local factor has been introduced to explain why calcification occurs only in one small area of the bone. Robison(7) favored the concept that there is a shift in pH toward the alkaline in regions where calcification is occurring. However, it has been shown that in cultures of embryonic chick femurs more calcification occurs at pH 7.0 than at 8.0(8). This occurs despite the facts that the pH of chicken plasma is 7.8 to 8.0(9) and that the optimal pH for chick bone phosphatase in the test tube is 9.85(10). In addition, bones growing on litmus have occasionally been observed to produce a distinctly reddish color in the region where calcification is occurring. This finding agrees with the report by Rous(11) that the pH of bone in the living animal is 6.5 to 7.2. It has also been suggested that the local factor is a system comprising phosphorylase, glycogen and phosphate ions, whereby the amount of substrate upon which phosphatase can act is increased. We are cognizant that phosphorylase may be active in our preparations but as yet we have been unable to test the action of alizarin upon it.

Let us consider the apparent discrepancy between the results obtained with alizarin and those obtained with litmus. Although both dyes have an affinity for calcium, concentrations of alizarin which do not retard the clotting process can stop calcification in tissue culture; concentrations of litmus which retard the clotting process will not stop calcification. The difference in calcification is probably related to the availability of the calcium ion and the solubility of calcium alizarinate compared to the calcium-litmus complex. Since calcium in combination with alizarin is much less soluble than is the calcium-litmus complex, one would expect a concentration of calcium alizarinate in an area where the calcium ion is most available. This would be within the zone of mineralization if one is willing to accept the not unfounded idea that in this zone there is a drop in pH which is favorable to the increased ionization of calcium. In the case of bones growing in tissue culture, this localized binding of calcium by alizarin has the effect of stopping the process of calcification. The calcium-litmus complex does not produce the same stoppage because it is much more soluble.

The clotting effects obtained with the two dyes are not contradictory in light of our calcification studies. They indicate that, in the medium in general, a higher concentration of calcium ions is necessary for clotting than for calcification. With litmus, clotting is interfered with at a dye concentration which does not inhibit calcification. With alizarin, calcification is stopped at a concentration of

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8. Paff, G. H., PROC. SOC. EXP. BIOL. AND MED, 1948, v68, 288.
9. Cameron, G., *Essentials of Tissue Culture Technique,* Farrar and Rhinehart, New York, 1935, p64.
10. Motzok, I., *Biochem. J.*, 1950, v47, 193.
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dye which does not affect clotting because the dye from the medium in general is concentrated within the zone of mineralization.

Our investigation has not found bone phosphatase to be an important factor in alizarin inhibition of calcification. In conjunction with this finding, Siffert(12), in a publication appearing after the completion of this work, reported finding phosphatase activity associated with preosseous cellular metabolism and not with the process of calcification.

Summary. Alizarin red S has been found to stop calcification without inhibiting the enzyme phosphatase either *in situ* or when added to solutions containing the enzyme extracted from bone. It has been shown that alizarin inhibition of calcification is not due to removal of the calcium ion from the medium in general as evidenced by the facts that there is no interference with the clotting process of plasma or with the action of embryonic hearts. Supporting evidence is presented from a study of bones growing on litmus-containing medium and from bones stained with alizarin and transferred to alizarin-free medium. An explanation of alizarin stoppage of calcification based upon the idea that locally there is a drop in the pH is advanced.

Received May 3, 1951. P.S.E.B.M., 1951, v77.

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Adenosine Triphosphate Levels in Mouse Blood after Whole-Body Irradiation and During Tumor Growth.* (18787)

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Whether the excessive release or depletion of high-energy adenosine polyphosphates is associated with pathogenesis has only recently been amenable to quantitative tests. Two pathological states in mice, both overtly characterized by wasting, were investigated in this regard: (1) that produced by whole-body X-ray irradiation; (2) that produced by a growing sarcoma implant.

Irradiation. Methods and procedure. Rockland Swiss male mice of 20-22 g in groups of 10 were simultaneously irradiated with a dual-tube X-ray machine.† A whole-body dose of 800 r was chosen as best suited to the experiment, as at this dosage a weight loss and general wasting was clearly evident after 48 hours (Fig. 1); deaths began on the

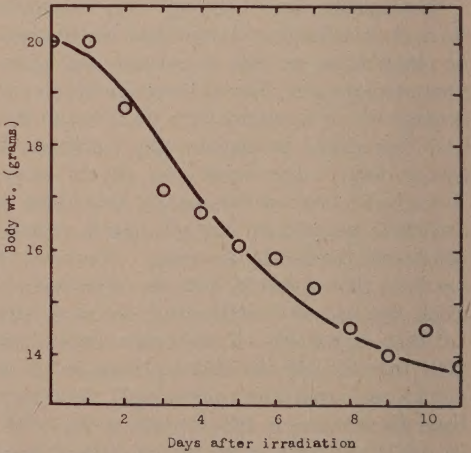

FIG. 1. Mean Body Wt of 20 Male Mice Following a Whole-Body X-ray Dose of 800 r, Plotted Against Time.

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* This project was supported in part by the U. S. Atomic Energy Commission, Contract No. AT(30-1)-863.

† Made available for our use through the courtesy of the Physics Dept., Memorial Hospital, New York City. KV—180; MA—25; distance from targets to mice—30 cm; delivery—480 r/min; diameter of aluminum-grill mouse container—16.6 cm; depth of container—3 cm.

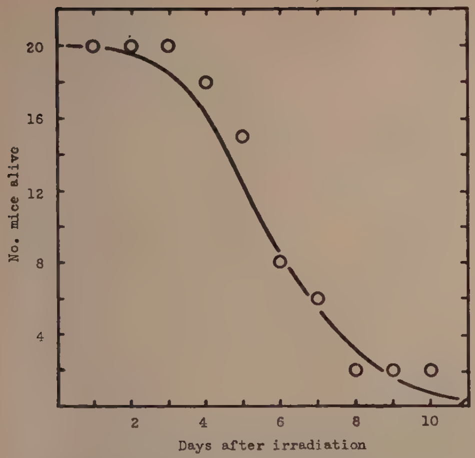

FIG. 2. Typical Survival Curve of 20 Male Mice, Following a Whole-Body X-ray Dose of 800 r.

4th day (Fig. 2), and all the mice were dead by the 11th day following irradiation. In one experiment a dose of 1000 r was used. Animals were sacrificed at various times after irradiation; hematocrit and nucleotide determinations were made on their blood. The technic for blood removal was as follows: the mice were anesthetized with parenterally injected nembutal, and the thoracic cavity was opened to expose the heart. Cardiac puncture was made with a No. 27 hypodermic needle into whose syringe 0.02 ml of an aqueous 5% heparin solution had previously been drawn. Usually about 1 ml of blood was withdrawn from each mouse, although in the advanced stages of irradiation sickness a diminution of total blood meant that less blood could be withdrawn than from non-irradiated mice. In these cases, enough mice were sacrificed from the same group to yield a total blood pool of 5 ml, the heparin content not exceeding 1 mg/ml. Neither nembutal nor the heparin, by test, interfered with the accuracy of the nucleotide assay. The analyses were carried out with 1 ml aliquots of blood, which were immediately deproteinized with 4 volumes of cold 2% perchloric acid. The protein precipitate was washed with an additional volume of cold 2% perchloric acid. The combined supernatant solutions, collected after centrifugation, were adjusted to pH 7 and made to a final volume of 10 ml. In some experiments, the cell sediment, or the serum withdrawn after centrifugation, were deproteinized and a protein-free filtrate prepared in similar fashion. Total nucleotide, and known nucleotide (adenylic acid, AA; adenosine diphosphate, ADP; and adenosine triphosphate, ATP) were assayed spectrophotometrically according to the method of Albaum and Lipshitz(1). In brief, total nucleotide was determined from the density reading at 2600 Å. The known nucleotide (AA, ADP, and ATP) levels were determined with specific enzymes. ADP and ATP were converted to AA and the latter in turn to inosinic acid in accordance with the following reactions:

$$(1)\ \text{AMP} \xrightarrow{\text{adenylic acid deaminase}} \text{Inosinic acid}$$

$$(2)\ 2\text{ADP} \xrightarrow{\text{Myokinase}} \text{ATP} + \text{AMP}$$

$$(3)\ \text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate}$$

This procedure has already been used for the measurement of these compounds in human blood(2).

Results. The alterations in mean body weight plotted as a function of days after irradiation is shown in Fig. 1. It is evident that a sharp decrease in weight begins about 24 hours after irradiation. Blood samples were assayed for total and known nucleotides at intervals of 2, 3, 4, 5, 6, and 8 days after irradiation. No consistent changes in either total nucleotide or in individual known nucleotides, as compared to non-irradiated controls, were observed. Nor did hematocrit levels change significantly during this period. The data for all the runs are shown in Table I. As indicated in the lower portion of Table I, such differences as occurred in the blood of irradiated and control mice, regarding total nucleotide and known nucleotides, are without statistical significance. Similar experiments carried out on red cells alone and serum alone revealed no significant differences between irradiated and non-irradiated mice.

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TABLE I. Total Nucleotide and Known Nucleotide Content of Whole Mouse Blood from Irradiated and Control Animals. The experimental values for mice sacrificed at varying times (see text) after irradiation showed no significant differences, and are therefore presented below in summated form. The figures in the "Known nucleotide" columns may be considered substantially to represent the ATP levels in the blood under test, for most of the known nucleotide was present as ATP. In only a few cases were AA and ADP detected, and their trace appearance showed no significant relation to one animal group or the other.

	Control group		Irradiated group	
	Total nucleotide	Known nucleotide (combined AA, ADP, ATP)	Total nucleotide	Known nucleotide (combined AA, ADP, ATP)
No. of mice	39	39	34	32
Mean value: mg %	13.3	7	12.8	6.1
Range	10.6–17.2	4.7–10.5	8.6–17.9	2.9–9.4
Stand. dev.	1.68	1.27	2.36	1.89
S. E. mean	.27	.20	.43	1.06
Coeff. variab.	12.7%	18.1%	19.2%	30.9%

Total nucleotide: S. E. difference .55
d–control group, exper. group .5

$$\text{d/S. E. } \frac{.5}{.55} = .91 \text{ (no significance)}$$

Known nucleotide: S. E. difference 1.07
d–control group, exper. group .9

$$\text{d/S. E. } \frac{.9}{1.07} = .84 \text{ (no significance)}$$

	Control group	Irradiated group
% packed cells in blood. Mean*	42.5	39.4

* Range of individual values approximately the same for both groups.

Sarcoma growth. Methods and procedure. During the course of the experiments described above, exploratory observations were made also on the nucleotide levels in the blood of mice bearing implanted tumors. Preliminary results appeared to justify more extended experiments in this direction. Accordingly, large groups of Rockland Swiss male mice, 20-22 g, were implanted with sarcoma 180, using a trocar technic(3) for subcutaneous deposition of the tumor tissue fragment in the axillary position. Groups of such tumor-bearing mice were sacrificed at daily intervals from the time of implantation to 17 days. Blood was taken under the same conditions as for the irradiated mice described above; and the same assay procedure for total and known nucleotides was followed. Recorded also at time of sacrifice were hematocrit, hemoglobin, body weight, and wet tumor weight.

Results. The alteration in body weight from the time of tumor implantation to the time of sacrifice is shown in Fig. 3. It is apparent that during the first 10 days there was no significant wasting as a result of tumor implantation and growth. The increase in tumor weight as a function of time is shown in Fig. 4. Tumor weight increased markedly from about the third day to the 12th, and then decreased. It was also observed that at about the 12th post-implantation day, many tumors ulcerated and much of their mass was ingested by the animals, leaving an open shell which was often cast off or resorbed. Such ingestion undoubtedly accounts for the drop in tumor weight during this period; while toxemic effects of the necrotizing tumor may well account for the body weight loss during this period. It should be pointed out that ulceration and tumor ingestion do not occur in all the mice. Many tumors after the 12th day

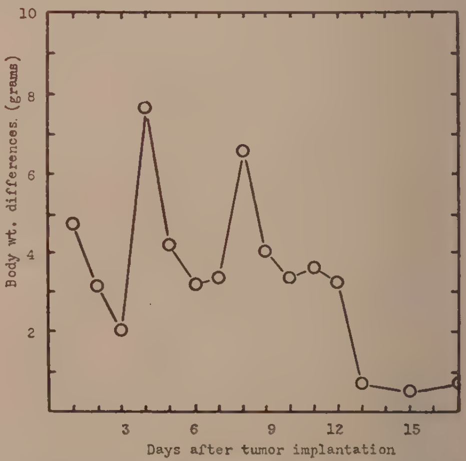

FIG. 3. Body Wt Changes in Mice Following Tumor Implantation. Points represent the differences between the mean body wt for all the mice (75) at the beginning of the experiment and the mean for each group of 5 sacrificed daily for blood determinations.

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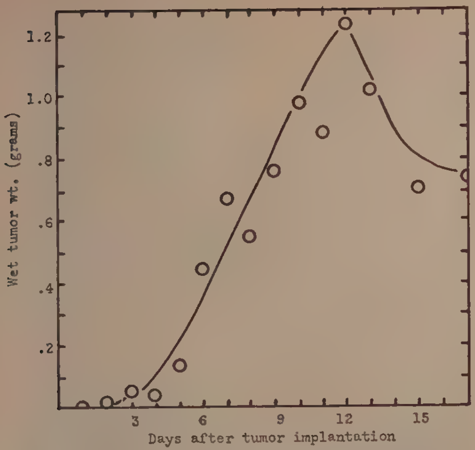

FIG. 4. Wet Wt of Tumors Plotted Against Days After Implantation. Each point represents the mean for 5 animals.

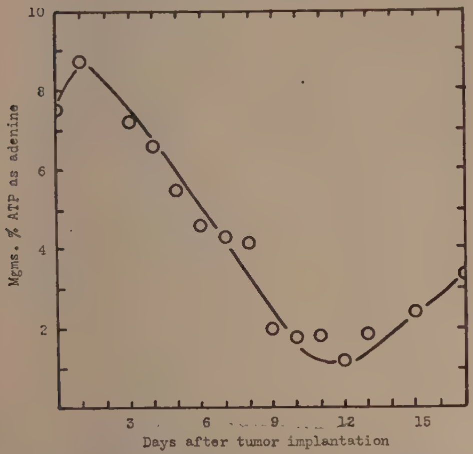

FIG. 5. ATP in the Blood of Mice Sacrificed at Daily Intervals Following Tumor Implantation. Each point represents the mean for 5 animals.

grow to massive proportions before the animal succumbs. The drop in the tumor weight curve after the 12th day obviously reflects the mean weights of both the partially ingested tumors and the non-ingested large ones.

No significant change was observed in the total nucleotide blood level during the observed 17 days of tumor growth. These data are not presented because their range and mean values were not significantly different from those in Table I for the irradiated and control mice. As to known nucleotides, there were only occasional trace amounts of AA or ADP, and these traces showed no consistent relation to one animal group or another. The bulk of the known nucleotide material was in the form of ATP and is presented as such in Fig. 5. After the first post-implantation day there appears to be a slight rise in blood ATP, followed by a sharp decrease which parallels the increase in tumor weight for this period. It appears, further, that the ATP level of the blood begins to increase again at the time when the tumor and whole body weights begin to decrease.

ATP in the blood is normally found in the red cells, not in the plasma. One may therefore argue that the decrease may be related to the hematocrit. That there is no relationship between blood ATP and hematocrit values until at least the 10th day is shown in Fig. 6. At the 10th, 11th, and 12th days when the ATP is lowest, the hematocrits are not significantly different from what they were before tumor implantation; a significant drop in the hematocrit begins with the 13th day along with the drop in body weight. There is too much variation in the hemoglobin values (Fig. 7) to justify any correlation, although the drop after the 13th day appears to be a consistent one.

If blood ATP is related to tumor growth, as would be supposed from the data of Fig.

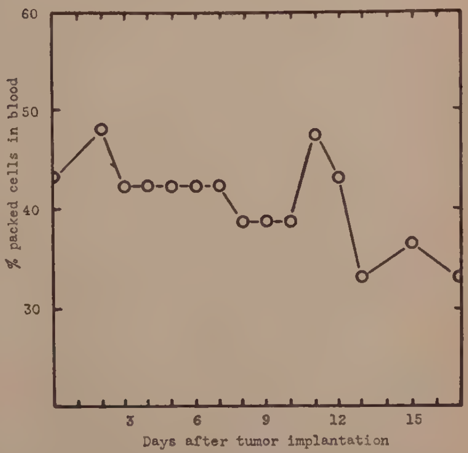

FIG. 6. Hematocrit Readings for Mice Sacrificed at Daily Intervals Following Tumor Implantation. Each point represents the mean for 5 animals.

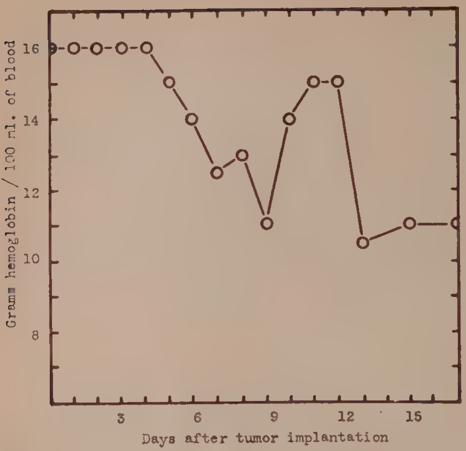

FIG. 7. Hemoglobin (Haden-Hausser) Readings for Mice Sacrificed at Daily Intervals Following Tumor Implantation. Each point represents the mean for 5 animals.

4 and 5, then tumor removal might also be expected to result in a rise of the level of blood ATP. This was tested. A technic was developed for removing five 10-day-old tumors without excision and with minimum trauma. This consisted of a single basal ligature which instantly stopped all vascular interchange between tumor and host body. Details of the technic are described elsewhere(4). One experiment consisted of extirpating 7-day tumors. After a recovery period of 2 days, the animals were sacrificed and their blood analyzed. Another experiment consisted similarly of extirpating 10-day tumors. The animals were sacrificed after a 5-day recovery period, and their blood analyzed. Not much alteration in ATP level occurred in the 2-day extirpation experiment, but considerable return toward the normal ATP level was apparent in the 5-day animals (Table II).

Discussion. We have observed no change in the nucleotide content of blood of mice undergoing post-irradiation wasting, a condition in many respects resembling shock. Green(5) reported a similarity between shock produced by injected myo-adenosine triphosphate and that produced by trauma. Meyer *et al.*(6) and McShan *et al.*(7) have proposed that the induction of shock may be favored by exhaustion of the energy store in blood and tissues represented by these purine derivatives. Kalckar and Lowry(8), on the basis of adenosine levels in the blood of traumatically shocked rabbits, concluded that adenylic acid components play no primary role in the etiology of shock; in addition, they found that the injection of adenosine deaminase, adenyl-pyrophosphatase, and alkaline phosphatases—materials expected to counteract any toxic action of ATP—did not alleviate traumatic shock.

In a further effort to illuminate the hypothesis that adenosine derivatives are shock-inducing, we injected ATP as sodium salt (purity: >90%) into mice. Parenteral doses of as high as 50 mg in 20 g animals failed to elicit overt symptoms of shock; indeed were wholly without observable toxicity. All mice so treated survived indefinitely. Intravenous doses of 20 mg were acutely lethal, but 10 mg doses were easily tolerated and did not induce shock-like symptoms. Higher parenteral doses were not attempted as being beyond the range of meaningful physiological experiment. These observations together with our failure to observe changes in the nucleotide content of blood of mice undergoing post-irradiation wasting, leads us to doubt that adenosine derivatives released into the blood stream by

TABLE II. ATP Levels in Whole Blood from Control Mice, Tumor-Bearing Mice, and Mice after Tumor Extirpation.

Type of animal	No. of animals	ATP mg % (mean)
Control	8	7.3
With 7-day tumor	7	5
With 7-day tumor; 2 days after extirpation	7	5.3
With 10-day tumor	8	3.4
With 10-day tumor; 5 days after extirpation	8	5.9

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injured tissues constitute a primary factor in shock, at least in so far as irradiated mice are concerned.

As to the marked ATP drop in the blood of tumor-bearing mice, little may at the present time be ventured in the way of interpretation. Whether this change in blood composition specifically reflects the tumor growth process, or whether it is merely a non-specific reaction to toxic materials released by a necrotizing lesion in the course of tumor growth, remains to be demonstrated. On such demonstration will depend the possible usefulness of our observation as a diagnostic tool. Also moot and as yet uninterpretable is the fact that total nucleotide levels during tumor growth do not change, whereas those of known nucleotide (ATP) do.

Summary. No changes were observed in the blood levels of total nucleotide or of adenylic acid, adenosine diphosphate, or adenosine triphosphate during the wasting period that follows whole-body X-ray irradiation of mice. Massive injected doses of ATP failed to induce overt symptoms of shock in mice.

In mice bearing implanted tumors there is a continuous decrease in blood adenosine triphosphate until about the 10th post-implantation day, followed thereafter for several days by a slight but significant rise in the ATP curve. The first phase of this curve parallels, on the time scale, the increase in tumor weight. Mice whose tumors were extirpated at stated intervals after implantation show a tendency to return to the normal ATP blood level. There is no apparent relation between changes in ATP blood levels and body weight, cell-fluid ratio in the blood, or hemoglobin, at least up to the 10th post-implantation day. No significant changes were observed in the total nucleotide values for blood during tumor growth; nor was the adenylic acid or the adenosine diphosphate picture different from that of normal mice.

Received May 14, 1951. P.S.E.B.M., 1951, v77.

Encephalitis in Midwest IV. Western Equine Encephalomyelitis Virus Recovered from Nestling Wild Birds in Nature. (18788)

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Neutralizing antibodies to the virus of Western Equine Encephalomyelitis (WEE) have been demonstrated many times in the sera of wild and domestic birds(1,2). This virus has been recovered only once from wild birds. Cox, Jellison and Hughes(3) isolated it from the brain and spleen of a prairie chicken shot August 27, 1941, near Rugby, N. D. The present paper reports 3 isolations of the WEE virus from the circulating blood of nestling wild birds in Weld County, Colo.

As part of an extensive search for the natural reservoirs of encephalomyelitis viruses, 1941 blood specimens were collected from nestling birds of 20 species (Table I). Collection of these was begun the first week of May and continued throughout the summer.

Technic of taking blood samples was as follows: Blood was obtained from nestlings 4 or more days old. A blood sample consisted of approximately 0.1 ml of blood withdrawn by cardiac puncture or from a wing vein into a syringe containing an equal volume of 10% inactivated normal rabbit serum in buffered saline. Each sample was placed separately

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† Virus and Rickettsia Section, Montgomery, Ala.

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2. Reeves, W. C., Proc. of the 49th Ann. Meet. of the U. S. Livestock Assn., p150. Dec. 1945.

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TABLE I. Nestling Birds' Bloods from Weld County, Colo. 1950 Tested for Virus.

	May		June		July		August		Sept.		Total		
Species	Nestlings	Nests sampled	Nestlings	Nests sampled	Nestlings	Nests sampled	Nestlings	Nests sampled	Nestlings	Nests sampled	Nestlings	Nests sampled	Positive nest samples
Pigeon	35	20	93	57	68	41	125	76	122	76	443	270	0
Barn swallow			7	2	91	22	45	11	10	4	153	39	0
Cliff swallow			32	11	108	46	36	15			176	72	0
Magpie	98	16	121	33	5	2					224	51	1
English sparrow	12	5	96	29	162	48	45	14			315	96	0
Yellow-headed blackbird			27	12	49	24					76	36	0
Red-winged blackbird			45	17	10	3					55	20	2
Other birds*	6	1	33	12	8	4	2	1			49	18	0
Total	151	42	454	173	501	190	253	117	132	80	1491	602	3

* Other birds included cormorant, great blue heron, marsh hawk, killdeer, avocet, mourning dove, long-eared owl, flicker, dipper, meadow lark, bullocks oriole, and bronzed grackle.

into a small tube and refrigerated immediately in a thermos flask containing wet ice. The tubes of diluted blood were sealed by means of an oxygen torch at the field laboratory and then stored on dry ice for shipment by air express to the Virus and Rickettsial Laboratory at Montgomery, Ala., to be tested for the presence of virus. The method of examining bloods for virus was as follows: Each blood sample was inoculated intracerebrally (0.02 ml) into 12- to 14-day-old white mice. No virus was recovered from any of the specimens except from the three mentioned earlier. The mice given the blood from these birds either died or became ill in 2 to 5 days. The brains of mice which died or showed symptoms of encephalitis were removed. Suspensions of these mouse brains were cultured and those free from bacteria were inoculated intracerebrally and intraperitoneally into 14-day-old mice, which either died or showed symptoms of encephalitis in 3 to 5 days. The 3 infective agents also proved to be pathogenic for guinea pigs.

After several serial passages in mice, identification of the viruses was undertaken. Each strain passed through a Seitz filter, was free from bacteria and was lethal to mice in a dilution of $10^{-7.6}$ to $10^{-8.7}$. By means of the neutralization test in mice each virus was found to be inactivated by antiserum of the Western equine encephalomyelitis virus, but not by antisera of the Eastern equine encephalomyelitis nor the St. Louis encephalitis viruses. The identification was completed by challenge inoculations of Western equine immune guinea pigs.

It was deemed noteworthy that the above procedures resulted in Western equine encephalomyelitis virus being isolated on 3 occasions from the blood of nestling wild birds obtained by the Encephalitis Investigations Unit working in Weld County, Colo. (Table I). Two of the bloods were from red-winged blackbirds *(Agelaius phoeniceus)*, 8 to 10 days old, and one was from a magpie *(Pica p. hudsonia)*, 21 days old. The red-winged blackbirds were from nests found near Nunn, Colo. and the infected blood samples were taken on June 26 and 30, respectively. The magpie was in a nest 10 miles south and 2 miles east of Greeley, Colo., and was bled June 29th.

The viruses differed slightly from the stock Western equine encephalomyelitis virus in having (a) a lower virulence for guinea pigs and (b) a slightly longer incubation period when first isolated, but otherwise serologically and immunologically similar to the stock Western equine encephalomyelitis strain.

Summary. Virus of Western equine encephalomyelitis was isolated on three occasions from circulating blood taken from nestling birds in Weld County, Colo. in June 1950.

Received May 18, 1951. P.S.E.B.M., 1951, v77.

Encephalitis in Midwest V. Western Equine Encephalomyelitis Virus Recovered from Mites *Dermanyssus americanus* Ewing. (18789)

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During routine testing for natural infection with neurotropic viruses, the virus of Western equine encephalomyelitis (WEE) was recovered from a collection of approximately 500 mites obtained from a nest of an English sparrow, *Passer domesticus,* in Weld County, Colorado, July 18, 1950, and identified as *Dermanyssus americanus* Ewing.

The mites were collected through a Berlese funnel, sealed in glass tubing, and while still alive, were frozen in dry ice and sent frozen to the Virus and Rickettsial Laboratory at Montgomery, Ala. for test. In the laboratory the mites were triturated in a sterile mortar with buffered saline containing 30% by volume of inactivated normal rabbit serum, diluted to a volume of 1.5 ml and the suspension centrifuged in a cold room at 13,000 rpm in an angle centrifuge. The supernatant fluid was then inoculated both intracerebrally (0.02 ml) and intraperitoneally (0.04) into 12- to 14-day-old white mice. The brains of mice which died or showed symptoms of encephalitis were removed, tested for bacteria, and suspensions of them inoculated intracerebrally into fresh mice.

An agent was thus isolated which had the following characteristics: it passed through a Seitz filter pad, was free from bacteria, was lethal to mice in a dilution of $10^{-6.5}$ to $10^{-8.5}$ and was neutralized by an antiserum of WEE virus, but not by that of the Eastern equine encephalomyelitis or the St. Louis encephalitis viruses. The identification of the virus as that of the Western equine type was completed by the challenge inoculation of guinea pigs previously immunized against this virus.

The sparrow nest was taken from an open cattle shed on a farm where a human case with symptoms of encephalitis had recently occurred. The patient was a 14-year-old boy who became ill on July 7, 1950. Antibodies neutralizing the Western equine encephalomyelitis virus were demonstrated in the serum withdrawn from the patient July 11th, the neutralization index being 50. A later serum sample drawn August 24th had an index of only 36, however, and did not indicate infection with Western equine encephalomyelitis virus since a rising titre of antibodies was not demonstrated.

Heart blood from a 9-day-old nestling occupant of the nest from which the virus-positive mites were taken was tested for the presence of virus, but none was isolated. Furthermore, no virus was recovered from the combined heart bloods of five 12- to 14-day-old nestlings from an additional English sparrow nest in the same building, nor from a collection of about 125 *D. americanus* infesting the nest.

The virus recovery here reported was the only isolation of a neurotropic virus in tests of

TABLE I. Mite Lots from Birds' Nests, Weld County, Colo. Tested for Presence of Neurotropic Viruses, May-August, 1950.

From nest of:	*Dermanyssus americanus*	*D. gallinae*	*Liponyssus sylviarum*	*D. americanus* and *L. sylviarum*	*D. americanus* and *D. gallinae*	*D. gallinae* and *L. sylviarum*	Total
English sparrow	28[*]	3	2	8	1		42
Barn swallow	5	10	2		1	3	21
Pigeon		9		1		1	11
Magpie	1		1				2
Northern cliff swallow		1					1
Double-crested cormorant	1						1
Total	35	23	5	9	2	4	78

[*] WEE virus isolated from one lot, July 18, 1950.

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[*] Encephalitis Investigations Unit, Office of Midwestern CDC Services, Kansas City, Kan.

[†] Virus and Rickettsia Section, Montgomery, Ala.

a total of 78 lots of *Dermanyssus* and *Liponyssus* mites from wild birds' nests collected in Weld County, May through August, 1950. The bird nest sources of the mites and the species tested are presented in Table I.

The tests were made on approximately 500 mites sealed for each nest, except where this number was not available, as at the first part of the nesting season. Mite species determinations were based on samples of usually 10 to 30 specimens preserved in alcohol from each nest. The methods of collecting and of testing as described above in the recovery of WEE virus were used in all tests.

Identification of the mites from the virus-infected lot was made on 34 specimens, all *D. americanus*, 6 males, 26 females and 2 nymphs. This is the first definite recovery of a neurotropic virus from *D. americanus*, although Reeves and his associates(1) obtained evidence of a possible infection of this mite with WEE virus in Kern County, California, where, in recoveries of the virus from *Liponyssus sylviarum*, they found *D. americanus* included with *L. sylviarum* in one pool of mites found infected with WEE virus. Natural infections with this virus have been reported from the chicken mite *Dermanyssus gallinae* by Sulkin(2) and from the tropical fowl mite *Liponyssus bursa* by Sulkin and Izumi(3).

Summary. Virus of Western equine encephalomyelitis was isolated from mites *(Dermanyssus americanus)* collected in Weld County, Colo. in July 1950.

Grateful acknowledgment is given to Assistant Sanitarian (R) John E. Lane of the Entomological Section, Laboratory Services, CDC, Atlanta, Ga. for identification of the mites.

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2. Sulkin, S. E., *Science*, 1945, v101, 381.

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Received May 18, 1951. P.S.E.B.M., 1951, v77.

Recovery of Virus of Eastern Equine Encephalomyelitis from a Mosquito, *Culiseta melanura* (Coquillett)* (18790)

Roy W. Chamberlain, Harry Rubin, R. E. Kissling, and M. E. Eidson.
(Introduced by Morris Schaeffer.)
From the Communicable Disease Center, Public Health Service, Federal Security Agency, Atlanta, Ga.

Although the virus of eastern equine encephalomyelitis has been transmitted by several species of *Aedes* mosquitoes in the laboratory(1,2) and is generally considered to be arthropod-borne, isolations from arthropods in nature have been few. Howitt *et al.*(3) made the first isolation of EEE virus from wild-caught arthropods when they recovered it from common chicken mites (*Dermanyssus gallinae* (De Geer)) and chicken lice (*Eomenacanthus stramineus* (Nitzsch)), collected in Tennessee in 1947. The only other isolation of EEE virus from arthropods in nature has been from a mosquito, *Mansonia perturbans* Walker in Georgia, also reported by Howitt *et al.*(4).

Western equine encephalomyelitis, on the other hand, has been recovered from wild-caught *Culex tarsalis* Coquillet(5), *Culiseta inornata* (Williston), *Culex pipiens* Linnaeus and *Anopheles maculipennis freeborni* Aitken

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* This note constitutes part of an investigation on encephalitis in Louisiana conducted by the Virus and Rickettsia Section, Montgomery, Ala.

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(6), *Aedes dorsalis* (Meigen) and *Culex stigmatosoma* Dyar(7), *Triatoma sanguisuga* Le Conte(8), *D. gallinae* (De Geer)(9), *Liponyssus bursa* (Berlese)(10), and *Liponyssus sylviarium* (Canestrini and Fanzago)(11).

This note reports the isolation of EEE virus from another species of mosquito, *Culiseta melanura* (Coquillett). The infected lot consisted of two specimens collected by light trap in the Manchac Swamp near Ponchatoula, Louisiana, on August 17, 1950. It is of interest that these specimens were taken within a few hundred yards of the location where a purple grackle with EEE virus in its blood was shot June 19, 1950(12).

The specimens were removed alive from the light trap, sealed in a tube, and kept frozen on dry ice until processed. At the laboratory in Montgomery, Ala., they were identified, triturated in 1 ml of tryptose phosphate broth, and spun 20 minutes in the cold at 4000 RPM in an angle-head centrifuge. After adding 2 mg of streptomycin and 500 units of penicillin, a portion of the supernate was used to inoculate six 3-week-old Swiss mice intracerebrally. Neurological symptoms were observed in the mice late the second day, followed by death on the third day. No bacterial growth was observed in broth or on blood agar plates following inoculation with suspension of brain material from the dead mice.

A second intracerebral passage caused death of mice in 2-3 days. Brains from this passage had an LD_{50} titer of 10^{-8}, as calculated by the method of Reed and Muench, upon intracerebral inoculation into mice(13). The infectious agent passed a Seitz EK filter.

Serum from a rabbit immune to the Decuir strain of EEE neutralized 3000 mouse LD_{50}'s of the new agent when serial 10-fold dilutions of infected brain were mixed with equal quantities of undiluted serum, incubated 18 hours at 4°C and inoculated intracerebrally into 3-week-old mice. WEE immune serum failed to have a neutralizing effect in tests conducted simultaneously. Sera from rabbits immunized against the new agent had a neutralization index of 1000 against the Decuir strain of EEE.

Guinea pigs immune to the Decuir strain of EEE resisted an intracerebral challenge of 10^7 mouse LD_{50}'s of the new agent. Those immunized against the new agent resisted a similar intracerebral challenge of the Decuir strain. Similarly immunized animals succumbed to 10^5 LD_{50}'s of WEE virus given intracerebrally.

Host range studies conducted in 10-day-old and adult mice, guinea pigs, hamsters, rabbits, and chickens revealed clinical courses similar to those of the equine encephalitides in these species. A pathological picture consistent with that produced by the arthropod-borne encephalitis viruses was found upon microscopic examination of brain sections of infected mice, guinea pigs, hamsters, and rabbits.

Confirmatory isolation from the original mosquito suspension was obtained by its reinoculation into a second group of mice.

As very little is known of the adult habits of *C. melanura* and only a single isolation has been made, the possible significance of this species in the epidemiology of EEE cannot be evaluated at the present time.

Summary. The virus of eastern equine encephalomyelitis was isolated from mosquitoes (*Culiseta melanura* (Coquillett)) collected by light trap in Louisiana, August, 1950.

Grateful acknowledgment is made to members of the Louisiana State Health Department and Department of Wildlife and Fisheries for their interest and assistance.

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Received May 18, 1951. P.S.E.B.M., 1951, v77.

Recovery of Virus of Eastern Equine Encephalomyelitis from Blood of a Purple Grackle.* (18791)

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(Introduced by Morris Schaeffer.)

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Although investigators have proved numerous avian species to be susceptible to eastern equine encephalomyelitis virus by experimental inoculation(1-6) and have reported its recovery from the brains of naturally infected birds(6-10), attempts to isolate the virus from other tissues of birds infected in nature have failed(10). However, Cox *et al.*(11) reported the isolation of western equine encephalomyelitis virus from the spleen and brain of a prairie chicken, *Tympanuchus cupido americanus* (Reichenbach).

This note reports the isolation of eastern equine encephalomyelitis virus from the blood of an apparently well adult male purple grackle, *Quiscalus quiscula quiscula* (Linnaeus), shot in flight in the Manchac swamp area near Ponchatoula, La., on June 19, 1950. Intracerebral inoculation of its blood into six 3-week-old mice was followed by death in five of the mice on the third day. No growth was observed following culture of the brains from these mice in dextrose or serum broth and on blood agar plates. The agent passed a Seitz EK pad. Infected mouse brain material had a titer of 10^8 LD_{50} upon intracerebral inoculation into mice, as calculated by the method of Reed and Muench(12).

Serum from rabbits immune to eastern equine encephalomyelitis virus gave a neutralization index of 15,000 against this agent when serial 10-fold dilutions of the agent were mixed with equal quantities of undiluted serum, incubated for 18 hours at 4°C, and inoculated intracerebrally in 0.03 ml amounts into 3-week-old mice. Western equine encephalomyelitis and St. Louis encephalitis antisera failed to neutralize in tests conducted simultaneously. Serum from rabbits immunized with this agent demonstrated strong neutralization of eastern equine encephalomyelitis virus. Guinea pigs vaccinated with the new agent resisted intracerebral challenge with 10^7 LD_{50} of eastern equine encephalomyelitis virus. Those immunized with eastern equine encephalomyelitis similarly failed to exhibit signs of infection upon challenge with an equivalent dose of the newly isolated agent.

In limited host range studies, the new virus resembled that of eastern equine encephalomyelitis both as to species susceptibility, and incubation periods. Microscopic examination of brains from infected mice, guinea pigs, and hamsters revealed a pathological picture consistent with that produced by the encephalitis viruses. Presence of the infectious agent in the original material was verified by reinoculation into a second group of mice with production of encephalitic symptoms on the second day, followed by death on the third day. On these bases, the agent was considered to be the virus of eastern equine encephalomyelitis.

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* This note constitutes part of an investigation on encephalitis in Louisiana, conducted by the Virus and Rickettsia Section, Montgomery, Ala.

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To our knowledge, this is the first report of the isolation of the eastern variety of equine encephalomyelitis virus from the blood of a naturally infected wild bird. It is of added interest that the specimen was collected during a period when there were comparatively few cases of the disease reported in horses in the region.

Summary. The virus of eastern equine encephalomyelitis was isolated from the blood of an adult purple grackle collected in Louisiana in June, 1950.

Received May 18, 1951. P.S.E.B.M., 1951, v77.

A Rapid Assay for Xanthurenic Acid in Urine. (18792)

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In man(1) and other mammalian species (2-6), a deficiency of pyridoxine has been shown so to alter the normal metabolism of tryptophan as to result in the urinary excretion of xanthurenic acid (XA). Following the ingestion of tryptophan, the quantitative measurement of XA in urine has been used as an index of pyridoxine deficiency or dysfunction(1). Colorimetric methods available at present for the determination of XA in urine generally involve preliminary time-consuming extraction of XA from urine prior to colorimetric assay(3,7,8).

In view of the increasing significance of pyridoxine in disorders of pregnancy(9-11) and possibly other clinical states(12-14), a rapid, reliable method for the estimation of XA in human urine would be of definite advantage for clinical studies where a large number of routine assays are to be performed. It is the purpose of this paper to present such a method.

Reagents and apparatus. 1. Five % sodium bicarbonate solution, freshly prepared prior to analysis. 2. $FeNH_4(SO_4)_2 \cdot 12H_2O$ solution. 0.85 g of Mallinckrodt analytical reagent grade, dissolved in 50 ml water. 3. Sodium hydrosulfite powder (J. T. Baker Chemical Co.). 4. Standard xanthurenic acid* solution: Stock standard solution, 500 μg per ml of XA, made by dissolving 25 mg of XA in 50 ml of 5% ethyl alcohol which contained 0.1 ml of concentrated ammonium hydroxide.

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* The xanthurenic acid used in these studies was synthesized in our Division of Organic Chemistry by Mr. A. Mebane and Dr. W. Oroshnik. This substance has a melting point of 297-298°C. An elementary analysis revealed the following: $C_{10}H_7NO_4$: Calculated. C, 58.54; H, 3.44; N, 6.83. Found. C. 58.40; H, 3.65; N, 6.66; Ash, 0.

This solution was stable for at least one month when kept under toluene in the refrigerator. The working standard solution, 100 μg per ml, was made by diluting 4 ml of the stock standard to 20 ml with distilled water. This solution should be prepared daily. 5. The Evelyn colorimeter was used with a 620 mμ filter.

Procedure. The urine was adjusted between pH 7.3-7.5 and then filtered through No. 1 Whatman filter paper. When XA assays were performed on a urine specimen following the administration of a test dose of tryptophan(1) the urine was diluted with water to contain 25-100 μg of XA per ml; it was found unnecessary to dilute specimens obtained before the ingestion of tryptophan. From 1 to 4 ml of urine (undiluted if small quantities of XA were present) were measured into each of two calibrated Evelyn tubes. To one, there was added 1 ml of the working standard containing 100 μg of XA to determine the XA recovery value; to the other tube (the actual test specimen) no standard XA was added. The contents of both tubes were diluted to 9 ml with water. To each tube, there was added 10 to 15 mg of sodium hydrosulfite, contained on the tip of a spatula, and this was followed by 0.10 ml of 1.7% $FeNH_4(SO_4)_2 \cdot 12H_2O$. It is important that the addition of the hydrosulfite precede the addition of the ferric ion in order to prevent the formation and subsequent precipitation of ferric hydroxide. The contents of the tube were then thoroughly mixed by gentle agitation. One ml of 5% sodium bicarbonate solution was now added to each tube. After gentle mixing so as to prevent the oxidation of the hydrosulfite by air, the blank values of both tubes were determined as described below. The colorimeter was first adjusted by setting the galvanometer at 100 with an aqueous solution containing 100 μg of pure XA per 10 ml assay tube which served as the reagent blank. This solution was prepared with the same quantities of reagents employed in the urine tubes above, except that 0.025 ml in place of 0.10 ml of 1.7% $FeNH_4(SO_4)_2 \cdot 12H_2O$ was added to the tube. The galvanometer readings of the blank tubes, *i.e.* the color of the urine and reagents in the reducing medium were then determined in the photoelectric colorimeter. After obtaining these readings, the contents of each tube were shaken well to permit oxidation until the green ferric-XA complex salt was formed and remained stable. Galvanometer readings were again made. All galvanometer readings were then converted to "L" values (Evelyn), and calculations were carried out in the conventional manner. Several points are worthy of note. The color was found to fade rapidly if the hydrosulfite was not completely oxidized. On the other hand, the color was stable for a period as long as 3 hours if destruction of the reducing agent was complete. When pure XA was analyzed, the excess iron slowly precipitated as the hydroxide. This resulted in a gradual loss of color. Thus it was necessary to read solutions of pure XA within 10 minutes after color was developed. Such precipitation did not occur in urine.

Results. The values obtained for urine and for standard solutions of XA were reproducible on different days. The color obtained obeyed Beer's law for solutions ranging from 10-250 μg of XA; a calibration curve was required when larger amounts of XA were measured.

The reliability of the test was examined by determining recovery values of XA added to urine specimens containing varying quantities of inherent XA. Thus at one extreme normal non-pregnant women have been shown to excrete very little urinary XA after a test dose of tryptophan; pregnant women close to term in certain pathognomonic states and pre-eclamptic women excrete large quantities of urinary XA under the same conditions(15). Typical data are presented in Table I.

Effect of time, temperature and pH. In the urine assay, the color reached a maximum immediately after oxidation of the hydrosulfite and remained stable in the daylight for at least 3 hours at room temperature. The color of the complex salt was stable between 15 and 37°C. The color intensity of the ferric-XA compound was at a maximum between

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TABLE I. Recovery of Xanthurenic Acid Added to Urine.

Urine No.	Amt urine assayed, ml	Urinary XA, μg/ml	Added XA, μg	XA recovered, μg	% recovery
1	4	3	0		
1	4		25	19	76*
1	4		50	40	80*
1	4		100	100	100
1	2	2	0		
1	2		25	26	104
1	2		50	49	98
1	2		100	92	92
2	1	126	0		
2	1		10	11	110
2	1		25	30	120
2	1		50	58	116
3	.2	457	0		
3	.2		100	109	109
4	.2	316	0		
4	.2		100	110	110

* When small quantities of XA are recovered in presence of large volumes of urine, recovery values are apt to be low because of interfering urinary pigments.

pH 7.0 and 8.2; it dropped off sharply on either side of this optimum range. It was essential, therefore, that before analysis the urine be adjusted between pH 7.3 and 7.5. Finally, the assay solution was buffered with bicarbonate, since an excess of hydrosulfite (which was not added quantitatively) brought the pH below 7.0.

Choice of buffer. Attempts to utilize more efficient buffers such as potassium bicarbonate and disodium phosphate in place of sodium bicarbonate were ineffective; the phosphate buffer gave rise to the formation of the insoluble ferric phosphate at pH 7.8. A 25% solution of potassium bicarbonate at room temperature lost carbon dioxide rapidly; this resulted in the formation of the highly alkaline potassium carbonate.

Specificity of reducing agents. The choice of reducing agent was found to be restricted to sodium hydrosulfite. Attempts to use other reducing agents such as ascorbic acid, sodium sulfite, and sodium bisulfite were unsuccessful. Compared to sodium hydrosulfite, the lower reducing potentials of these substances would explain these findings. It should be noted that excessive quantities (greater than 20 mg) of sodium hydrosulfite cannot be used in this procedure, for under such conditions the acidity of the assay solution might be so increased as to prevent the return to the desired pH range 7.0-8.2 upon the addition of sodium bicarbonate.

Discussion. It could be demonstrated by paper chromatographic methods and absorption spectrum studies(16) that the green color resulting on the addition of ferric ion to urine was due *specifically* to XA. Although the exact chemical nature of the Fe-XA complex remains yet to be determined, the amount of ferric ion used in this assay was well beyond the critical concentration necessary for optimum color development. This was established as follows: When a given amount of XA was titrated with increasing amounts of ferric ion, the maximum color intensity occurred in a ratio of one mole of ferric ion to 2 moles of XA. In the procedure described herein, approximately 10 times this amount of ferric ion was added to all tubes containing urine. This was necessary since urine is known to contain chelating substances which combine with iron and thereby render it unavailable for coordination with XA.

Summary. A colorimetric method for the direct estimation of xanthurenic acid in urine without preliminary extraction is described. The method is based on the reduction of the ferric to ferrous ion by sodium hydrosulfite to obtain a reliable blank value and the subsequent oxidation of the reactants to obtain the green ferric-xanthurenic acid complex salt. Provisions are made for the use of an internal recovery standard with each sample of urine assayed.

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Received May 21, 1951. P.S.E.B.M., 1951, v77.

A New Human Blood Factor of Rare Incidence in the General Population.
(18793)

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On studying the serum of an Rh positive mother, Mrs. R. M., para 4, gravida 4, who delivered an infant suffering from severe hemolytic disease, an antibody was observed which reacted with the blood of the father, the oldest sibling, and the affected infant, but not with the bloods of the remaining 2 siblings. The factor was present in the blood of the infant's paternal grandmother, but not in his grandfather, nor in the blood of his father's 2 siblings. Thus, the agglutinogen was demonstrated in the blood of 4 out of 10 members of 3 generations of this family.

This new blood factor has a remakably low incidence in the general population since not one reaction was observed in tests of 425 random individuals of groups O and A with the serum of the immunized mother.

With the permission of the affected infant's parents, the new agglutinogen was designated as the Miltenberger blood factor. The abbreviated forms for the gene, factor, and antibody may be represented as Mi^a, Mi^a, and anti-Mi^a, respectively. The corresponding allelic factor whenever it may be demonstrated can be referred to as Mi^b. The mother of the affected infant had 3 children, all normal at birth and now living and well. The fourth infant, born at term on January 27, 1951 was very pale and somewhat jaundiced. Because of the appearance of dyspnea and more intense jaundice 12 hours after birth, the infant was transferred to the Children's Hospital, where a replacement transfusion with Rh negative blood was carried out. The blood count and hemoglobin values on admission, 2,120,000 and 8 g respectively, were increased to 5,600,000 and 15.2 g. The infant was readmitted at the age of 5 weeks for an additional transfusion and at present there is complete recovery.

Serologic tests on the infant's family with standard antibodies follow:

			Tests with anti-sera				
			D	C	E	c	Mi^a
Father	O	MN	0	0	0	+	+
Mother	A	M	+	+	0	+	0
1st child	A	M	+	+	0	+	+
2nd ”	O	MN	+	+	0	+	0
3rd ”	A	M	+	+	0	+	0
Affected infant	O	MN	+	+	0	+	+
Paternal grandfather*	B		+	+	0	+	0
” grandmother	O		+	+	0	+	+

* Absence of the factor in this blood and also in that of the father's two siblings was determined by tests with neutralized anti-Mi^a.

Both parents were positive for Fy^a (Duffy), S, and negative for K (Kell) and Le^a (Lewis). The mating was incompatible for the factors Lu^a (Lutheran), Jk^a (Kidd)(1), and N, but antibodies for these factors could not be demonstrated.

Significant results, however, were obtained on testing the mother's serum with the father's red cells. Titration at 37°C with saline suspended cells showed a value of 1:2-1:4, but more distinct effects were found in the indirect anti-globulin test, which showed a titration value of 1:16-1:32. As in the case of anti-Fy^a, the reaction could not be elicited in tests with trypsinized cells(2). The direct Coombs reaction on the infant's cells carried out on the fifth day of life was very weak but this test was performed after a replacement transfusion with group O, Rh negative and presumably Mi^a negative blood. It is safe to assume that the direct Coombs test must have been much stronger at delivery.

The further study of this serum was facilitated by the fact that the only atypical antibody present was anti-Mi^a. This was demonstrated in a series of tests at 18°C with numerous bloods of all antigenic varieties. Under these conditions weak reactions were

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1. Allen, F. H., Diamond, L. K., and Niedziela, B., *Nature*, 1951, v167, 402.

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obtained only with bloods known to possess the Mi^{a} factor.

Titrations of anti-Mi^{a} with the indirect anti-globulin technic on the four positive bloods of the Miltenberger family showed uniform values of 1:16-1:32. Thus, the double-dose effect indicating homozygous individuals could not be demonstrated, but this was not surprising since all four individuals are obviously heterozygous. As is to be expected on the basis of gene frequencies of any factor with such low incidence in the general population, bloods homozygous for the factor must be exceedingly rare, and by the same token the allelic factor Mi^{b} should be present in almost all bloods tested. Whether or not Mi^{a} is genetically related to either of the two factors characterized by unusually high incidences, Tj^{a} (Jay)(3) or k (Cellano)(4) is still to be determined.

Three blood factors of low incidence (Levay)(5), Gr(6), and Jobbins(7) occurring mainly in members of particular families have been reported recently, but their possible relationship to each other and Mi^{a} cannot be determined because of lack of antibodies for simultaneous studies.*

In contrast to the antibodies for the 3 factors mentioned above, anti-Mi^{a} is the only one held to be responsible for hemolytic disease and hence is clinically important. Both anti-Mi^{a} and anti-Gr are the only atypical antibodies present in their respective sera, but they differ from each other since anti-Gr is probably of the naturally occurring variety, its origin by antigenic stimulus of pregnancy or transfusion having been excluded.

The occurrence of antibodies for factors such as Mi^{a} makes it necessary to exclude their presence in the serum of all instances of hemolytic disease presumed to be due to ABO incompatibility. In this latter group of cases, antibodies reacting only on occasional bloods can be excluded if absorption of the mother's serum with a number of bloods of group A (or B) removes all activity for the father's blood of group A (or B). Only then can one consider the possibility that isoimmunization with resulting hemolytic disease was brought about by the action of anti-A or anti-B.

Summary. A new blood factor, Mi^{a}, demonstrable with the aid of an immune isoantibody responsible for hemolytic disease of the newborn is briefly described. Its hereditary nature is indicated by its presence in four out of 10 individuals in three generations of a particular family, in contrast to its absence in 425 random bloods.

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3. Levine, P., Bobbitt, O. B., Waller, R. K., and Kuhmichel, A. B., Proc. Soc. Exp. Biol. and Med., 1951, v77, 403.

4. Levine, P., Wigod, M., Backer, A. B., and Ponder, R., *J. Hemat.*, 1949, v4, 869.

5. Callendar, S. T. and Race, R. R., *Ann. Eugenics*, 1946, v13, 102.

6. Graydon, J. J., *Med. J. Australia*, 1946, v2, 9.

7. Gilbey, B. E., *Nature*, 1947, v160, 362.

* According to Race, anti-Levay cannot be identical with anti-Mi^{a} since the latter antibody failed to react with blood known to contain the Levay factor. Tests of anti-Mi^{a} with bloods positive for the Jobbins and Gr factors are still to be made.

Received May 22, 1951. P.S.E.B.M., 1951, v77.

Isoimmunization by a New Blood Factor in Tumor Cells. (18794)

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In the course of preparing a 66-year-old female patient, Mrs. D. S. J., for gastric resection of a tumor later diagnosed as adenocarcinoma, difficulties were encountered in selection of compatible donors.* The patient was in group O, Type MNS, presumably of the Rh genotype DCe/DCe (R_1R_1), Duffy and Cellano positive. The factors P, Lewis, and Lutheran could not be demonstrated in her blood. Among 3000 random group O individuals, all bloods were hemolyzed by the unheated serum or agglutinated by the inactivated serum. The hemolysin could also be demonstrated in the patient's inactivated serum by adding sensitive red cells suspended in pooled fresh group compatible unheated serum from normal individuals. The unusually high incidence of positive reactions is apparently attributable to a single antibody since all activity is removed after treatment of the serum with each of 10 different bloods.

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* The initial observation on an atypical antibody in the patient's serum was made by Dr. O. B. Bobbitt.

These observations suggested the presence of a new hereditary factor which is present in the red blood cells of almost all individuals tested. For identification purposes, the blood factor and its antibody may be designated as "Jay" and "anti-Jay" respectively. Because of the relationship to the tumor described below, the abbreviated symbols for the gene, blood factor, and antibody are Tj^a, Tj^a, and anti-Tj^a, the letter "T" referring to the tumor. The corresponding symbols for the theoretical allelic gene, factor, and antibody can be designated as Tj^b, Tj^b, and anti-Tj^b, respectively.

Tests of anti-Tj^a with the blood of her 3 siblings, all in group O, showed that the blood of one of her sisters did not contain the factor, but it was present in the red cells of the sister's 4 children.[†] The factor could be demonstrated in the blood of the patient's husband and their 4 children, all in group A, since their red cells absorbed anti-Jay.

The titer of the antibody, 1:8, when first studied increased to a titer of 1:512 as result of a so-called biologic test, *i.e.* an intravenous injection of 25 cc of a random sensitive group O blood. This was followed by an immediate severe reaction and her grossly pink serum, obtained 15 minutes later, contained 180 mg % free hemoglobin.

The gastric tumor, a grade II adenocarcinoma invading the muscularis, was uneventfully removed by a gastric resection and transfusions were not required. It is significant that the mucosa over the tumor was not ulcerated. The tumor was finely divided and when lyophilized was found to be more or less suitable for absorption experiments. Remarkably enough, 20 mg of the dried tumor specifically absorbed 16-32 units of the antibody. These experiments were properly controlled since under the same conditions the dried tumor cells failed to diminish the activity of anti-D, although the D antigen is present in the patient's red cells. The tumor cells also failed to diminish the activity of anti-A, anti-B, or anti-E. No activity could be demonstrated in concentrated saline extracts, and the tumor cells after extraction retained their affinity for the antibody. Unfortunately normal gastric tissue was not available to test for the presence or absence of the factor.

As in the case of Levine and Stetson(1)—an intragroup hemolytic reaction in a pregnant woman at the very first transfusion—the main interest here lies in the source of the immunization. The last pregnancy had occurred 32 years previously, and there is no history of previous transfusions or hemolytic disease in any of her four children. Since the tumor cells contain the antigen, one may assume that they are also responsible for the isoimmunization. Apparently, the tumor cells contain the Jay antigen which is present in the form of a blood factor in the red blood cells of almost all individuals except the propositus and her sister.[‡]

If the Jay factor is inherited, its presence and absence are determined by two genes of equal dominance as in the case of the MN, Rh-Hr, and Kell-Cellano systems. Since the factor is not present in the patient's blood, her genotype may be represented as Tj^bTj^b and paradoxically she has been harboring, at least in her tumor cells, the Jay gene Tj^a not transmitted from either of her two parents. In the absence of external sources for the blood factor in the tumor cells, one is led to assume that its unexpected presence may be brought about by intrinsic changes in the body, *i.e.*, a genetic mutation.

[†] The antibody could not be demonstrated in the sister's serum. Since submitting the manuscript, an antibody of identical specificity *i.e.* anti-Jay was found in the serum of a patient, Mrs. Mc, living in South Africa. In this case, the antibody was of the saline variety (titer 1:128) and the antigenic stimulus was probably derived from four pregnancies, each of them ending in spontaneous abortions after 3 or 4 months. With both sera, there were only 3 bloods (Mrs. Jay, her sister and Mrs. Mc.) which were not agglutinated. These findings will be reported by Zoutendyk and Levine.

1. Levine, P. and Stetson, R. E., *J.A.M.A.*, 1939, v113, 126.

If only one locus—the site of the gene for the blood factor—is affected, then the mutation may be held responsible for the presence of the blood factor in the tumor. Because mutations have been frequently discussed in the pathogenesis of malignancy, one is tempted to suggest that the mutation may have also induced the tumor. This question, however, must be left open, in view of the importance of the issue involved and the absence of any direct evidence.

In any event, the highly exceptional circumstances in the case here reported provided specific agglutination reactions, the interpretation of which has led to a rather unexpected conclusion that a blood factor in tumor cells may arise from a genetic mutation.

[‡] For production of immune isoagglutinins resulting from transplantable tumors in mice and rats cf. Gorer(2) and Lumsden(3). Isoimmunization by tumor cells is also a possible explanation for the origin of a very weak anti-Duffy which caused a hemolytic transfusion reaction at the first transfusion of a 64-year-old patient suffering from benign prostatic tumor. This case was recently reported by Rosenfield, Vogel, and Race(4) who made no comments on the origin of a presumably immune antibody in the pre-transfusion specimen.

2. Gorer, A. P., *J. Path. and Bact.*, 1937, v44, 691.
3. Lumsden, T., *Am. J. Cancer*, 1938, v32, 395.
4. Rosenfield, R., Vogel, P., and Race, R. R., *Rev. D'Hem.*, 1950, v5, 315.

Received May 22, 1951. P.S.E.B.M., 1951, v77.

Excretion of Coxsackie Virus (Conn. No. 5 Strain) in the Urine of Infected Mice.* (18795)

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In the already abundant literature on infection with viruses of the Coxsackie group, we have found but one casual reference to the recovery of virus from the urine of human cases(1). Howitt, in listing the sources from which Coxsackie virus had been recovered includes urine from one case. No details are given. There have been no reports concerning the elimination of virus in the urine of infected animals. Because of the possible epidemiologic importance, the following observations demonstrating viral excretion in the urine of infected mice seem worth placing on record.

Exp. I. Several 0-day-old mice were inoculated intraperitoneally with 10^{-3} dilution of Conn. No. 5 passage virus in buffered saline. After 48 hours, at which time the mice showed the usual signs of illness, urine was obtained from several animals by direct aspiration of the bladder with a tuberculin syringe. The urine was kept frozen in the syringe for 5 days, then thawed, and penicillin and streptomycin (500 units per cc) added. The final dilution was approximately 1 to 3. Ten mice, 1 to 2 days old, were inoculated intraperitoneally with 0.02 cc of this diluted urine. Three days after injection, 9 of the 10 mice were dead or missing; the moribund survivor was sacrificed, and a 10% suspension of carcass, to which 500 units of penicillin and streptomycin per cc had been added, was frozen for further passage. Liver and pancreas were examined histologically and showed the usual lesions found in suckling mice infected with the Conn. No. 5 strain

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* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Howitt, B. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 443.

of Coxsackie virus(2). The 10% suspension of carcass of the suckling mouse, from which skin, feet, tail and gastrointestinal tract had been removed, was diluted in 15/100 M phosphate-NaCl buffer to 10^{-3}, and inoculated into nine 0-day-old mice. All were dead on the 4th day.

Exp. II. Again, Conn. No. 5 passage material, in dilution of 10^{-2}, was inoculated intraperitoneally into 3 mixed litters of 0-day-old mice. After 48 hours, urine was collected by aspiration of bladder with a syringe. The urine, to which penicillin and streptomycin had been added, was diluted 1 to 10 with phosphate-saline buffer solution and frozen. After 4 days, the urine was thawed, and 0.02 cc of 10-fold dilutions injected intraperitoneally into 0-1-day-old mice. All mice given the highest dilution—10^{-5}—succumbed to the infection. Histologic examination of mouse No. 4394, killed when moribund on the 6th day after injection of 10^{-3} dilution showed interstitial pneumonia and early necrosis and calcification of cervical fat.

A second titration of urine was made to determine the end-point, with the results shown in Table I.

TABLE I. Infectivity of Urine for Suckling Mice.

Dilution	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	8/8*	8/8	6/9	2/9	1/9	1/9	1/9

* Numerator—No. of dead mice; denominator—total No. of mice inoculated.

The I.D.$_{50}$ as calculated by the Reed-Muench method, was $10^{-5.6}$. The virus is thus shown to be present in high concentration in the urine.

Controls. Eight 0-day-old mice were inoculated intraperitoneally with 0.2 cc of urine obtained from normal 2-day-old mice, killed by decapitation. One mouse was found dead on the following day, but showed no microscopic lesions. Another was missing on the 3rd day. A healthy survivor, sacrificed on the 6th day, had normal organs. The 5 surviving mice remained well.

Presence of virus in the urine of adult mice infected with Conn. No. 5 virus. Although adult mice have been regarded as not being susceptible to infection with the Coxsackie group of viruses, it has been found in this laboratory that the Conn. No. 5 strain regularly produces severe lesions in the pancreas, and that the virus can readily be maintained in adults by serial passage of pancreas suspensions. A full account of these observations will be published elsewhere(3). It was, therefore, thought of interest to ascertain whether this virus might also appear in the urine of infected adults. Accordingly urine was collected on the 4th day after intraperitoneal inoculation of female mice averaging 15.6 g in weight at the beginning of the experiment. All the animals had lost weight, and were proven to have pancreatic disease when killed. This was the 4th passage of the virus in adult mice. Nine 1-day-old mice were inoculated intraperitoneally with 0.02 cc of urine. Five mice survived for 22 days, without showing signs of illness. One mouse, found dead on the 5th day, was not examined. Two were sacrificed for histologic study. One of these was killed on the 5th day. No characteristic lesions were found. The second mouse, however, when sacrificed on the 11th day, had marked tremors and was obviously sick. There was found diffuse encephalitis and myelitis; the lesions were identical with those in the central nervous system of Conn. No. 5 infected sucklings surviving for a corresponding period. Also there was extensive necrosis with calcification in the cervical and interscapular fat pads.

A second experiment confirmed the fact that virus may at times be excreted in the urine of infected adult mice. Pooled urine from 11 young adult mice was collected on the 4th, 6th and 7th day. They had been inoculated intraperitoneally with 0.1 cc of 10^{-1} suspension of carcass of infected sucklings, and had shown when killed the usual weight loss and gross pancreatic lesions. The 4-day specimen was contaminated, and the mice injected with it died of bacterial infection. The urine obtained on the 6th day failed to produce characteristic signs and lesions, when

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2. Pappenheimer, A. M., Daniels, J., Cheever, F. S., and Weller, T. H., *J. Exp. Med.*, 1950, v92, 169.

3. Pappenheimer, A. M., Kunz, L. J., and Richardson, S., *J. Exp. Med.*, in press.

inoculated into new-born mice. The specimen secured on the 7th day, however, was found to contain virus, all 10 of a litter dying or being sacrificed when showing obvious illness. Three of them were proven, on histologic examination, to have typical lesions of myocardium, liver and pancreas. A fourth was frozen, and after 10 days storage in the deep freeze, proved infective when injected intraperitoneally in 10^{-1} dilution into 0-day sucklings. All the animals died on the 3rd day. Three examined histologically proved to have characteristic pancreatitis and hepatitis.

This experiment proves that Connecticut No. 5 virus may be excreted in the urine of infected adult mice. The optimal period and concentration remain to be determined.

Exp. III. That the lesions produced by inoculation of urine from infected mice are due to the Conn. No. 5 virus and not to nonspecific toxic substances is indicated by the fact that the addition of immune anti-Conn. No. 5 serum to the infected urine for one hour before inoculation confers at least partial protection.

Infected urine	+ Conn. #5 antiserum	.02 cc	3/8
” ”	+ normal mouse serum	”	8/8
” ”	+ anti-Ohio R serum	”	8/8

The 3 deaths in the first group occurred within the first 2 days after injection and were probably non-specific. Similar early deaths have occurred in mice inoculated with normal urine.

Conclusion. Infant mice infected with the Conn. No. 5 strain of Coxsackie virus eliminate virus in the urine. The infectious titer of urine obtained on the 2nd day after intraperitoneal inoculation is $10^{-5.6}$. Adult mice with pancreatic disease caused by Conn. No. 5 virus may also excrete virus in the urine.

Received May 23, 1951. P.S.E.B.M., 1951, v77.

Production of Cytoplasmic Azurophilic Inclusions in Connective Tissue Cells by Suspending Agents.* (18796)

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During recent studies on the action of cortisone acetate (Cortone-Merck) on acute localized antigen produced inflammation in the mouse(1), it was observed that cytoplasm of fibroblasts of loose connective tissue of treated mice contained acidophilic inclusions (2,3). The purpose of this investigation was to obtain information on the origin and nature of the inclusions and on the mechanism underlying their formation.

TABLE I. Changes in Percentage Distribution of Fibroblastic Inclusions in Cells of Loose Connective Tissue of Intact Mice Produced by Cortone (Merck).

	No. of animals	Mean % of fibroblasts containing cytoplasmic inclusions and S. E. 4 hr	24 hr	48 hr	96 hr	6 days
1 mg Cortone s.c. in 2 cc suspending agent	20	28 ± 9.87	40.6 ± 6.28	36.6 ± 5.58	34.6 ± 4.59	26.5 ± 5.63
2 mg Cortone s.c. in 4 cc suspending agent	20	36.5 ± 7.15	52.5 ± 4.47	48 ± 4.87	42.6 ± 5.68	28.2 ± 4.65
1 mg crystalline cortisone acetate s.c. in .2 cc saline	20	0	0	0	0	0

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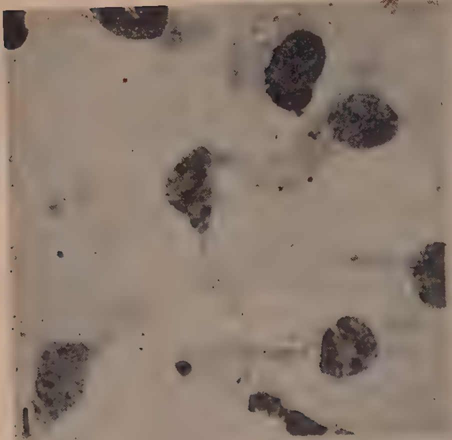
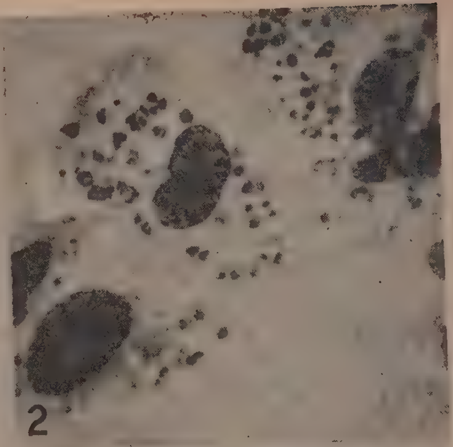
* Aided by grants from the Life Insurance Research Foundation and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

1. Dougherty, T. F., and Schneebeli, G. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 854.

2. Schneebeli, G. L. (Abst.). *Anat. Rec.*, 1950, v106, 78.

3. Schneebeli, G. L., Loewe, S., and Dougherty, T. F. (Abst.) *Anat. Rec.*, 1951, v109, 122.





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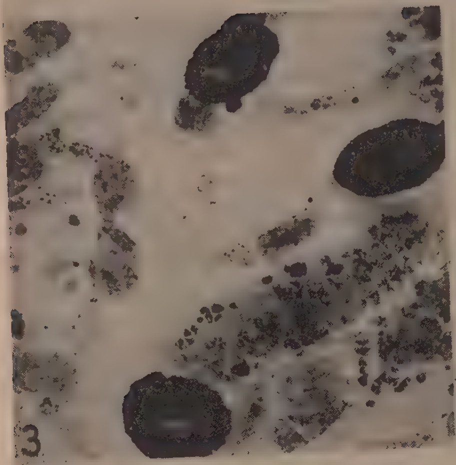

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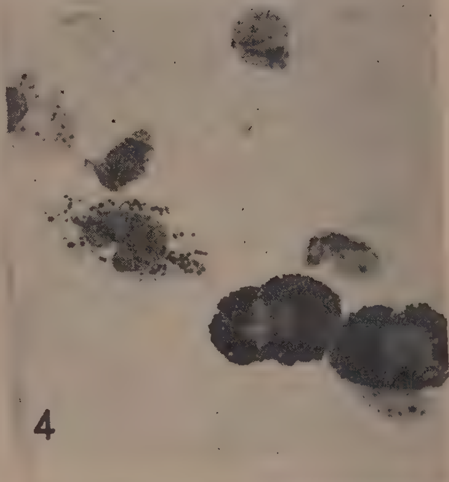

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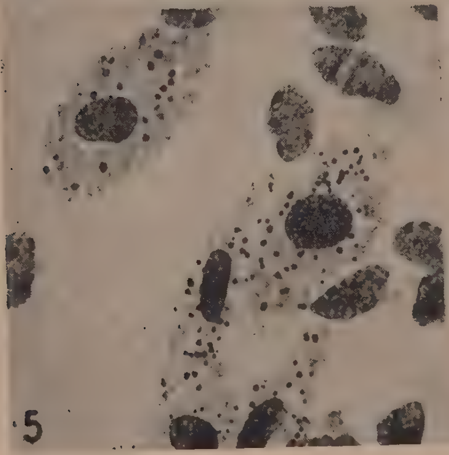

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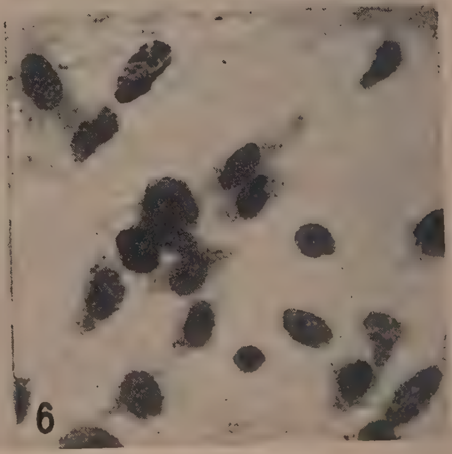

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Photomicrographs #1-6, Showing Spreads of Loose Connective Tissue of Non-sensitized Intact Animals. The magnification of Fig. 1, and 3-6 is ×720; Fig. 2 is ×830.

FIG. 1. Normal connective tissue.
FIG. 2. Connective tissue 4 hr after s.c. injection of 1 mg cortisone.
FIG. 3. Connective tissue 24 hr after s.c. injection of 1 mg cortisone.
FIG. 4. Connective tissue 48 hr after s.c. injection of 1 mg cortisone.
FIG. 5. Connective tissue 96 hr after s.c. injection of 1 mg cortisone.
FIG. 6. Connective tissue from the area of cortisone injection (4 hr after 1 mg).

Since Cortone is an aqueous suspension with stabilizing and suspending agents, the present work included a study of the role of both the hormone, and the suspending vehicles in respect to their capacity of inducing cellular inclusions.

Materials and methods. About 200 CBA mice of equal sex distribution, 16 weeks of age and of an average weight of 20 g were used. The animals were kept under optimal laboratory conditions with free access to food (dog chow supplemented with calf meal and vitamins) and drinking water.

The cellular inclusions were studied with the aid of the connective tissue spread method (1). The tissue sheets taken either from the site of injection or from other subcutaneous sites were expanded on slides to a thin cuticle, quickly air-dried and stained with May-Grünwald Giemsa. In some of the spreads, a differential cell count was performed. A total of 800 cells was counted in each of several spreads from the same animal, and the percentage of cells containing inclusions was determined for each cell type. The compounds employed for injection were Cortone (Merck) and the various components of the cortisone acetate suspending vehicle. Pectin, methylcellulose, polyvinyl alcohol and spinal cord lipids were other macromicellar materials studied for comparison with the components of cortisone suspending vehicle. The injections were made into the subcutis of the shaved abdomen if not otherwise indicated. The exact procedures employed are described below with the results.

Results. Cortisone acetate was injected subcutaneously in either 1 mg or 2 mg doses (Table I). Spreads were taken from the subcutaneous area of injection at 2, 4, 24, 48, 96 hours and 6 days after treatment. Single subcutaneous injections of either dose of Cortone were followed by accumulation of azurophilic cytoplasmic inclusions (Table I, Fig. 2 to 5), in fibroblasts of loose connective tissue in the injected area. The number of these inclusions varied from a few to 100 or more and their size averaged between 0.5 and 2 μ. There were marked variations in the shape of these inclusions within single cells (Fig. 2-5). The number of fibroblasts affected by 1 mg or 2 mg of Cortone was negligible 2 hours after injection, rose sharply in the subsequent 2 hours and moderately from the 4th to the 24th hour. At this time, the phenomenon reached its peak with the involvement of nearly 50% of the fibroblasts at the site of injection. After 24 hours the number of affected fibroblasts decreased, but so slowly that even after 6 days the incidence was still more than one-half of that found at 24 hours. Crystalline cortisone acetate (1 mg) suspended in saline did not produce the azurophilic inclusions at any of the periods studied. It did, however, damage fibroblasts which were in the area of deposition of the crystals. (Fig. 6).

The various components of the cortisone suspending agent were tested for their capacity to induce cytoplasmic inclusions. At least 5 animals were used in each group listed in Table II. Spreads of loose connective tissue were taken 6 hours after administration from the area of injection. The approximate number of cells containing cytoplasmic inclusions is indicated by pluses in Table II. As depicted in Table II, a cortisone acetate free solution containing all other components of Cortone (Components A, B, and C) was as effective in producing topical cellular inclusions as Cortone. On the other hand, no inclusions appeared after Component A (saline and benzyl alcohol) and only a comparatively small percentage of fibroblasts was affected when the injected solution contained "Tween 80" (Component B). This leaves carboxy-

TABLE II. Cellular Inclusions in Loose Connective Tissue of Intact Mice Produced by the Cortisone Acetate Suspending Agent and Other Colloidal Agents. (6 hr after s.c. injection.)

Cortisone acetate (in suspending agent) 1 mg in .2 cc	3+	Pectin .5% in .2 cc saline	3+
Crystalline cortisone (suspended in saline) 1 mg in .2 cc	—	Methylcellulose .5% in .2 cc saline	—
Cortisone suspending agent. Comps. A, B, and C,* .2 cc	3+	Polyvinyl alcohol .5% in .2 cc saline	—
Comp. A, .2 cc	—	Spinal cord lipids .5% in .2 cc saline	—
Comps. A and B, .2 cc	+	Saline sol., .2 cc	—
Comp. C, .2 cc	3+		

* Comp. A—saline sol. with 1.5% Benzyl alcohol; Comp. B—saline sol. with .4% Tween 80 (Polyoxyethylene sorbitan Monooleate); Comp. C—saline sol. with .5% sodium carboxymethyl cellulose.

TABLE III. Distribution of Fibroblast Inclusions in Mice Receiving .1 cc Cortone, Cortone Suspending Agent or Its Components. (Spreads taken 6 hr after injection.)

	s.c. injection					i.p. injection		
	Injection area		Incision area	General		Incision area	General	
Component tested	Intact	Adrex	Adrex	Intact	Adrex	Adrex	Intact	Adrex
Saline Sol.	0	0	0	0	0	0	0	0
Comp. A	0	0	0	0	0	0	0	0
A + B	+	+	0	0	0	0	0	0
C	3+	3+	+	0	0	0	0	0
A + B + C	3+	3+	+	0	0	0	0	0
Cortone + Comp. A + B + C	3+	3+	2+	+	+	2+	+	+

5 animals used in each experimental group. For constituents of components tested, see Table II. 1+ = 1% to 10%. 2+ = 10% to 40%. 3+ = more than 40%.

methylcellulose (Component C) as the major responsible factor. Carboxymethylcellulose differed from Cortone in that it produced a localized inflamed area in which azurophilic inclusions were found in macrophages as well as in fibroblasts. When carboxymethylcellulose was given along with cortisone acetate (Cortone) the local inflammatory reaction was inhibited and inclusions were confined to topical fibroblasts since macrophages were absent. Of the other macromicellar components tested, only Pectin was as effective in producing cellular inclusions (in fibroblasts as well as macrophages) as carboxymethylcellulose (Table II). No effect on connective tissue cells was observed in animals treated with methylcellulose, polyvinyl alcohol or spinal cord lipids.

In 120 mice, Cortone and the components of the cortisone suspending vehicle were tested for their capacity to produce cellular inclusions in areas remote from the site of injection. To exclude endogenous adrenal cortical secretion, which could be responsible for a possible generalized effect on the fibroblasts, 5 of 10 animals in each group were adrenalectomized prior to injection with the test component preparation. The components were injected subcutaneously or intraperitoneally into groups of 60 mice each. Connective tissue spreads were taken from various locations after 6 hours, as follows: (a) from the area of injection in subcutaneously injected mice; (b) from the incision area for adrenalectomy in adrenalectomized mice; and (c) from subcutaneous areas distant from the injection zone in the subcutaneously injected intact and adrenalectomized animals. In adrenalectomized subcutaneously injected mice, the cytological phenomena were qualitatively the same as in intact animals (Table III). The incidence of topical fibroblastic inclusions was high after subcutaneous injection of any solution containing carboxymethylcellulose, much lower when the solution contained only "Tween 80" and zero when it contained neither of these 2 agents. When carboxymethylcellulose was given with cortisone acetate (Cortone), fibroblasts in sites other than the local injection area contained azurophilic inclusions.

The number of fibroblasts affected when Cortone was administered to the adrenalectomized animals was smaller than that seen in intact mice in spreads taken from distant areas of the subcutis. Intraperitoneal administration of Cortone also produced generalized fibroblastic inclusions. No generalized inclusions were found after "Tween 80" or carboxymethylcellulose administration. In Cortone injected mice the incidence of distant fibroblasts containing inclusions was lower after intraperitoneal than after subcutaneous injection.

Morphological characteristics of the inclusions. Irregularly shaped small dark blue inclusions appeared in the cytoplasm of fibroblasts about one-half hour after injection. Their number and size increased greatly during the following 3-4 hours with accompanying changes in coloration. After 4 hours, the inclusions were large azurophilic elements with jagged edges. They differed from intracellular granules, *i.e.* those in mast cells, by their variable size, shape, number and staining characteristics; from the inclusions characteristic of phagocytes by their homogeneity and shape and by the absence of a surrounding vacuole. During the first hours the affected fibroblasts maintained their normal shape (Fig. 3). However, later the inclusions increased in number and size and the cells tended to assume a more rounded shape. After 24 hours there was a slow decrease in the number and size of inclusions in affected cells (Fig. 5).

Preliminary observations indicated that the azurophilic inclusions were not modified mitochondria. Also, it was apparent that they were not composed entirely of lipid since they did not disappear when treated for prolonged periods with high concentrations of ethanol.

Comment and summary. The evidence presented indicates that various suspending agents produce characteristic changes in fibroblasts and macrophages of loose connective tissue. Similar alterations occurred in endothelial system. The azurophilic inclusions were so named because they stain with the azur component of the polychrome dyes used. The data indicate that administration of cortisone acetate in conjunction with the suspending agents tends to increase the number of affected fibroblasts in areas remote from the site of injection.

The number of azurophilic inclusions in each fibroblast and their persistence for many days following a single injection suggests that the function of this cell may be altered. This possibility should be taken into account in evaluating the action of Cortone on connective tissue reactions. Further, since the suspending agents also affect endothelial cells it is conceivable that they (as well as cortisone itself) could be responsible for modifying capillary permeability as described by Menkin(4). Also, such agents might modify the rate of repair following traumatic or chemical injury by altering the fibroplastic activity of fibroblasts.

The alterations in the affected cells did not appear to be typically degenerative since their nuclei were not adversely affected except in the very central portion of the site of injection. The azurophilic material did not appear to be phagocytized substance but rather altered cytoplasm.

Although the presence of azurophilic inclusions has not been described by other authors, intravenous injection of polyvinylpyrrolidon for prolonged periods produced alterations in reticulo endothelial cells of spleen, lymph nodes, liver, etc., which were similar to those found in lipoid storage diseases(5).

In view of the extent and duration of the cytological alterations described here and the widespread use of suspending agents, further investigation of their possible harmful tissue effects should be pursued.

We are indebted to Dr. Augustus Gibson of Merck & Co., for the supply of Cortone and its cortisone acetate suspending agents.

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5. Ammon, R., and Müller, W., *Deutsche Med. Wchnschr.*, 1949, v74, 465.

Received May 24, 1951. P.S.E.B.M., 1951, v77.

Methods for Local Induction and Quantitative Analysis of Human Sweat. (18797)

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(Introduced by F. L. Horsfall, Jr.)
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The demonstration by Conn(1), that the concentration of sodium in the sweat is affected by the systemic administration of steroid hormones has given a new significance and immediacy to the quantitative study of sweat composition. When due allowance has been made for the influence of the rate of sweat flow upon the concentration of sodium, as in the standardized test of Locke *et al.*(2), it may be possible to use this phenomenon for the clinical evaluation of patients with suspected disturbance of steroid metabolism. Further progress in the exploitation of this system for pharmacological and clinical ends would seem to require a greater command of its physiology than is now available. Such a development, as contrasted with the rapid growth of renal physiology, for instance, has been delayed by want of convenient and quantitative means for the induction, collection, and analysis of sweat.

As a means of inducing the required flow of sweat, the superficial injection of a small dose of some cholinergic drug offers a number of advantages: (1) convenience, (2) freedom from general disturbance of physiological state, (3) quantitation and timing of the stimulus to the glands, (4) opportunity for comparison of the responses of symmetrical sites to different stimuli or of the same site to repetitive stimuli. This form of stimulation has been used in numerous studies, but the investigation of the glandular response apparently has been confined to the qualitative detection of emergent moisture. Quantitation of the response to local stimulation requires the use of special procedures for the collection and analysis of small quantities of sweat, since the advantages cited above would disappear in the attempt to induce sweating by this means over an area greater than a few cm^2. The capillary tube technic of Lobitz and Osterberg(3) could be used for this purpose, and would allow observation of the response of a small number of glands; however, for studies in which it is sufficient to determine the average behavior of a larger number of glands (100 to 1000, depending on the length of the collection period), the methods to be described appear to be both simpler and more exact. Actually, they are at least as simple as the classical methods of harvesting sweat in ml quantities from large areas of skin. Because they allow the complete collection of sweat from an anatomically homogeneous region and the subdivision of the collection into short, accurately timed periods, the present methods are definitely superior to the large scale collections even for the study of the sweat response to general stimulation.

Procedure. The procedures described in the present report combine local stimulation of the glands with quantitative analysis of the sweat. The flow of sweat is induced by the subdermal injection of a cholinergic drug in a dosage which is sufficiently small to make the systemic effect inappreciable, yet is massive with respect to the cells in the neighborhood of the injection. The sweat is absorbed into preweighed discs of filter paper (S & S No. 589, Green Ribbon, 25 mm diameter); the amount collected, usually between .010 and .100 ml per disc, is determined by the increment in weight. Subsequently, each disc is extracted for analysis of either sodium or urea; other substances of interest could be determined by appropriate methods of micro analysis. Evaporation is prevented during

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*Porter Fellow of the American Physiological Society; supported in part by the Rosenstock Memorial Foundation.

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2. Locke, W., Talbot, N. B., Jones, H. S., and Worcester, J., *J. Clin. Invest.*, 1951, v30, 325.

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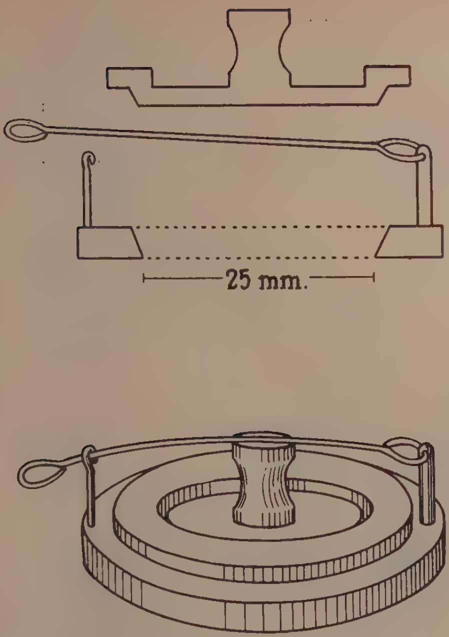

FIG. 1. Plastic Chamber for the Collection of Sweat.

the collection by a plastic unit, which consists of a Bakelite ring and a Lucite cover (Fig. 1). The important details in the design of this unit are the bevelled vertical junction between cover and ring, the flat overhang of the cover which is pressed tightly against the ring by an arc of spring wire, and the chamber of 1 mm depth that remains to accommodate the disc when the unit is closed. A satisfactory fixation of the ring to the skin may be obtained with a thin film of Duco Household Cement, providing that the ring is held firmly in place until the cement is dry, a matter of about 20 minutes. Once securely fixed, the ring may be left in place for at least 3 days. Before the test the required number of filter paper discs are placed in glass-stoppered weighing vessels (Pyrex, 30 ml capacity) and each vessel with its contained disc, is weighed to a precision of ±0.03 mg. Thereafter each disc remains enclosed in its own vessel, except for the time of collection. The vessels are kept covered with paper hoods, and are handled with care to avoid contamination of the rim or stopper. At the completion of a test the vessels are returned to the balance room, allowed to come to temperature equilibrium, and reweighed. If the stimulus is to be local, the test area enclosed by the ring is first irrigated with distilled water, dried with analytical grade filter paper and then left open to allow observation during the injection. A 25 gauge, 1½ inch needle is introduced into the skin outside of the ring and passed in a horizontal direction until its tip lies just under the skin at the center of the area. Through it the stimulating solution is injected in a volume of 0.1-0.5 ml. The needle is withdrawn, a disc quickly set in place and the unit closed. Thereafter, at intervals of 10-30 minutes for a total of 90-120 minutes, the discs are replaced.† In case it is desired merely to collect the entire output in one long period, it is convenient to employ two discs at the same time; the water holding capacities of about 0.10 ml per disc are additive under these conditions. For the most part Mecholyl (acetyl-β-methylcholine chloride) in a dose of 0.2-2.0 mg has been used to stimulate the sweat glands; comparable results have been obtained with Prostigmine methylsulfate (0.1 mg), and Furmethide (furtrethonium iodide, 0.2 mg). Although procaine alone does not cause the excretion of sweat under the conditions of this test, it potentiates the action of Mecholyl and in addition renders the injection entirely painless. For these reasons it has usually been included in the solution for subdermal injection: Mecholyl 25 mg, procaine 20 mg (2 ml of a 1% solution), and sterile water to 12.5 ml. A volume of 0.25 to 0.50 ml of this solution will provoke a brisk local sweat response in the average subject.

After the test is completed and the weights of the samples have been determined, the discs are extracted for chemical analysis. An extract, suitable for analysis of sodium with a Perkin-Elmer flame photometer (Model 52A), is given by the following procedure. Each vessel receives 25 ml of $LiNO_3$ solution (75

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† Water evaporates from a moist disc at about 1 mg/min; since transfer of disc from plastic chamber to closed weighing bottle can easily be completed within 2 seconds, evaporative loss may be neglected.

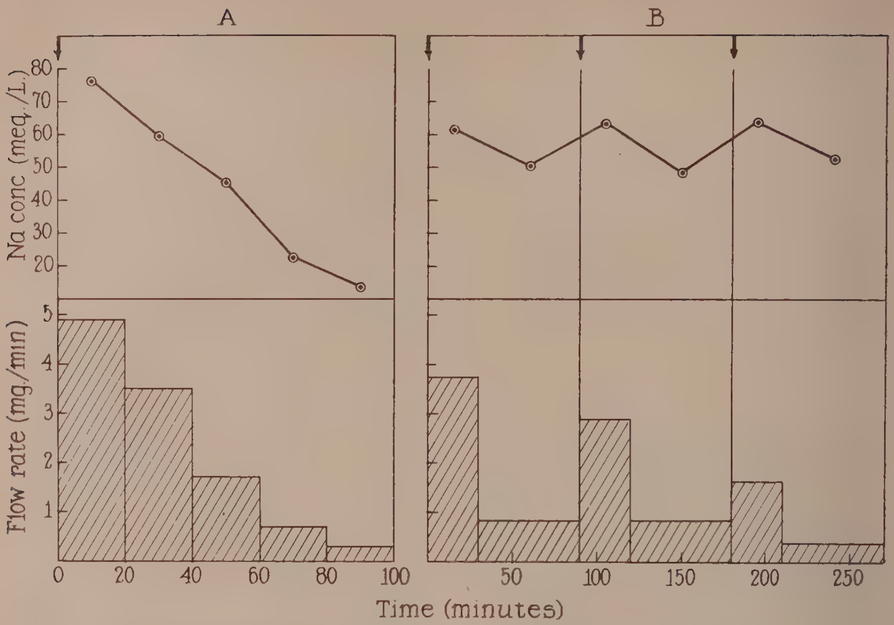

FIG. 2. The Local Excretions of Sodium and of Water Following a Single and Following Multiple Subdermal Injections of Mecholyl, 1 mg and Procaine, .8 mg. (Exp. A and B were performed on the same subject, but on different days).

meq/L); it is stoppered, shaken until the disc fragments, then decanted through porous filter paper (S & S No. 588—1H, 7 cm) to remove the pulp. Although complete extraction can be obtained without fragmentation of the disc when 12 or more hours are allowed for diffusion, the filtration cannot be avoided if the atomizer has a fine capillary, because a few fibers inevitably become detached from the disc. When the discs are to be analyzed for urea, rather than sodium, the extraction proceeds differently. To each vessel 0.1 *N* H_2SO_4 is added in volume of from 2 to 6 ml, depending on the estimated content of urea. The flasks are allowed to stand at room temperature overnight with occasional swirling, and then decanted to separate the extract from the disc. The determination of urea can be effected by a modification of the colorimetric method of Archibald(3). Aliquots of the extracts, 1 ml in volume, are pipetted into matched Pyrex cuvettes (10 x 75 mm size); equal volumes of standard solutions of urea in 0.1 *N* H_2SO_4 (6 to 24 γ/ml) and of 0.1 *N* H_2SO_4 alone are treated similarly to serve as standards and blanks, respectively. To each tube is added 1 ml of an acid mixture (conc. H_2SO_4, 180 ml; conc. H_3PO_4, 540 ml; water to a total volume of 1 liter), and 0.3 ml of a 3% solution of α-isonitrosopropriophenone in ethyl alcohol. The cuvettes are closed with rubber stoppers and shaken vigorously to mix the contents. In order to maintain the closure while the tubes are heated, a wide rubber band is stretched from the bottom to the stopper of each tube; this remains in place more securely if a small solid glass rod projects from the center of each stopper. After this preparation the tubes are immersed in a boiling water bath, from which light is excluded, and heated for 2 hours. They are then cooled to 20°C and their optical densities at 540 mμ determined in a spectrophotometer. From these data the urea concentrations of the unknown solutions are calculated in the usual manner.

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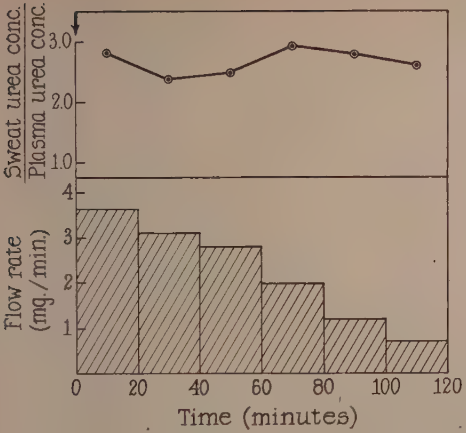

FIG. 3. The Local Excretions of Urea and of Water Following a Single Injection of Mecholyl 2 mg and Procaine 1.6 mg.

Success in application of these procedures of collection and analysis requires a meticulous chemical technic to avoid contamination; the glassware must be clean and be protected from dust in storage; all manipulations, except handling the outside of containers, must be performed indirectly with forceps or a clean glass rod. Even with great care in these respects, however, it was found that the blank value for sodium was excessive; this difficulty, which presumably arose from the extraction of sodium from glass surfaces, was eliminated by coating all glassware with silicone.[‡] With attention to all details, and with siliconed glassware, the blank values for sodium and for urea should be less than .2 μeq. and .5 γ, respectively. For comparison, the amounts of sodium and of urea contained in 10 mg of sweat with concentrations of these substances in a low normal range (30 meq./L and 500 mg/L, respectively) are .3 μeq. and 5 γ; this quantity of sweat approximates the smallest amount to which the present method may be usefully applied.

Results. Typical responses to local stimulation of the sweat glands are illustrated by the graphs (Fig. 2 and 3). It may be seen that the rate of sweat flow reaches an early maximum and then declines; that this decline is due both to removal of the stimulus and to fatigue of the glands is suggested by the fact that reinjection of the drug provokes another, but lesser, output of sweat. In more detailed studies on the rate of water excretion after a single injection, it has been found that the excretion commences within 5 seconds after the injection, rises to a maximum rate within the first 20 minutes, and thereafter declines exponentially with a half time of 10 to 30 minutes.

The concentration of sodium varies in parallel with the rate of flow; this relation is similar to that found with thermal sweating (*2*), and, because of the more intense stimulation obtained with local injection, is demonstrable over a greater range (Fig. 2A). It should be noticed, however, that it may be affected by the preceding functional activity of the glands, since different relations between concentration and flow rate are found with repeated injections into the same area; for instance, in Fig. 2B the concentrations are as high after the third injection as after the first, despite lower rates of flow from the area. Urea, unlike sodium, is excreted at a concentration which exceeds that of the plasma and which is independent of the rate of sweat flow over wide limits (Fig. 3); with rates of excretion which are at the lower limit of the present methods there appears to be a further increase in the concentration.

Summary. Procedures are described for the stimulation of sweat by local injection of a cholinergic drug, the quantitative collection of the output from a definite area during consecutive periods, the determination of the volume and of either the sodium or the urea concentration in each sample. In their present form, these procedures are applicable to quantities of sweat between 10 and 200 mg; with slight modifications of the methods, these limits could be extended.

Received May 24, 1951. P.S.E.B.M., 1950, v77.

[‡] Clean glassware by boiling in detergent solution, followed by boiling in sulfuric acid-dichromate cleaning mixture. Rinse thoroughly with distilled water; dry in oven. Expose surfaces to 2% solution of silicone (DC200, 350 Ctsk viscosity) in CCl_4; drain off excess. Dry at 100°C for 12 hours, then bake at 300°C for 30 minutes. This gives a durable coating, which remains indefinitely if exposure to detergents is minimized in subsequent cleanings.

Effectiveness of Thiocyanate Analog of Vitamin B_{12} in Pernicious Anemia.* (18798)

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Since the announcement of crystalline vit. B_{12}(1) two hemopoietically active modifications, B_{12a} and B_{12b}, have been described. Vit. B_{12} has been shown to be a cyano-cobalt coordination complex(2) and has been designated cyano-cobalamin(3). Vit. B_{12a} does not contain the cyano group(2). Comparative data now indicate vit. B_{12a} and B_{12b} are identical(4) and represent the replacement of the cyano group by an hydroxo group in the vit. B_{12} molecule(3). This substance therefore has been designated hydroxo-cobalamin(3). Other analogs of vit. B_{12} in which the cyano group of cyano-cobalamin has been replaced by other groups such as chloro, cyanato and sulfato have been prepared and tested for their microbiological activities(3). Vit. B_{12a}(4,5) and B_{12b}(6) had previously been reported to have a therapeutic potency in pernicious anemia comparable to that of vit. B_{12}. Studies on the effectiveness in pernicious anemia of other analogs of vit. B_{12} have not been reported. Buhs, Newstead, and Trenner(7) have prepared the thiocyanate analog of vit. B_{12} in which the cyanide ion of vit. B_{12} was replaced by the thiocyanate ion. Like vit. B_{12a} (hydroxo-cobalamin) it was found to be microbiologically equivalent to vit. B_{12} in the *L. lactis* cup assay but differed from vit. B_{12} in titrimetric assay methods(7). In the present paper we report studies showing the thiocyanate analog of vit. B_{12} to be fully active hemopoietically in pernicious anemia.

We reported previously(8) that certain biochemical changes can be observed preceding and during the hematologic response to crystalline vit. B_{12} in pernicious anemia. These include an early decrease in urinary excretion of phosphate, followed by increased uric acid excretion with beginning reticulocytosis, and increased phosphate excretion during the period of maximum reticulocytosis. In the present investigation similar biochemical studies were correlated with the hematologic and clinical responses to the thiocyanate analog of vit. B_{12}.

Two previously untreated white male patients with pernicious anemia in relapse were placed on a general hospital diet which furnished a constant intake of phosphorus (about 1.6 to 1.7 g phosphorus daily). Clinical diagnosis was substantiated by presence of a macrocytic anemia, megaloblastic bone marrow and histamine refractory achlorhydria. Crystalline thiocyanato-cobalamin, in saline solution, 15 μg per cc, was administered parenterally according to the schedule used with vit. B_{12}: 15 μg initially, then 5 μg daily

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* We wish to express our appreciation to Dr. William Branch Porter, for his permission to carry out this study on the Medical Service. A generous quantity of the analog was supplied us by Merck and Co., Rahway, N. J., through the courtesy of Dr. R. A. Peterman. We are indebted to Dr. N. F. Young for serum potassium determinations on the flame photometer, and to Anne Anderson, Mary Dodson, Vivian Wilkerson and Carolyn Williams for technical assistance.

† John and Mary R. Markle Foundation Scholar in Medical Science.

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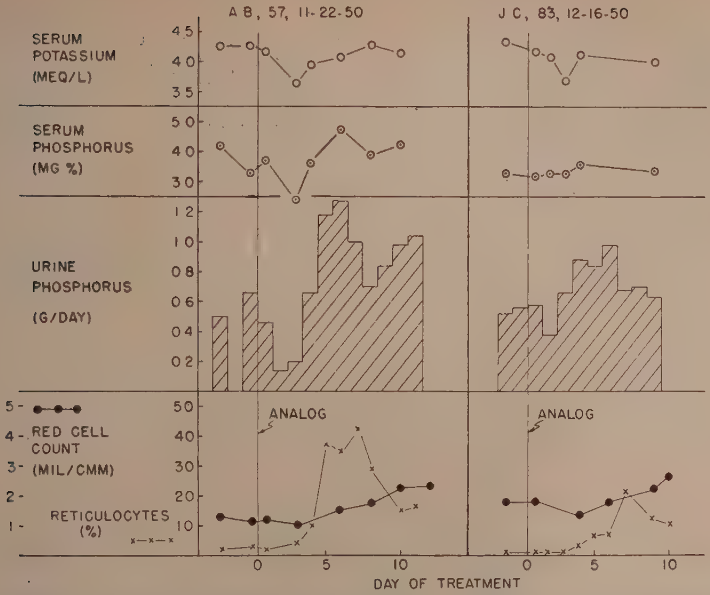

FIG. 1. Effects on Urinary Phosphorus, and on Serum Potassium and Phosphorus, with Relation to the Reticulocyte and Total Red Cell Response in 2 Cases of Pernicious Anemia Treated with the Thiocyanate Analog of Vit. B_{12}.

until the peak of the reticulocyte response after which 5 μg was administered every other day. The clinical response to treatment was similar to that observed by us with crystalline vit. B_{12} except for the neurological manifestations in the older patient. Striking in each patient was the remarkable effect on appetite, general strength and feeling of well-being. Patient A.B. (57 years) was discharged after 12 days treatment with the analog. He was then given vit. B_{12} since it was necessary for him to leave the city. During the period of hospitalization he received a total of 60 μg of thiocyanato-cobalamin in 10 days, an average of 6 μg per day. The only neurological finding was minor subjective tingling in the fingers, present for a week prior to admission, which he said improved following treatment.

Patient J.C. (83 years) was treated continuously with the analog for a period of 61 days and received a total of 225 μg, 75 of which were given during the 9 days of hospitalization.‡ Neurological symptoms were moderately severe and had been present for about 6 weeks. In general the neurological response was disappointing. After discharge he received a single dose of 15 μg per week for 2 weeks. The dose of the analog was then increased to 30 μg a week for 3 weeks without noticeable improvement. When our supply was exhausted (after 61 days) he was given vit. B_{12} and about a month later, after 90 μg, stated there was some subjective improvement.

Maximum reticulocytosis occurred in each subject on the 7th day of treatment, but response in the younger man, A.B., was more vigorous (Fig. 1). Increases in hemoglobin and hematocrit were consistent with improvement in the red cell count. Leucopenia (<3000) was present prior to treatment in both instances. In A.B. a normal leucocyte count was obtained on the 4th day of therapy, while in J.C. a moderate leucopenia (2350 to 4200) persisted until the 9th day of treatment after which normal counts were obtained.

Fig. 1 shows the response in urinary phosphorus excretion in both men. The effect was the same as that observed previously with crystalline vit. B_{12} administered in the same dosage. There was a prompt diminution in urinary phosphate excretion which occurred before any change in reticulocyte count. This was more marked in A.B. and was accompanied by a drop in serum phosphorus and serum potassium. Following the initial decrease there was a marked increase in urinary phosphorus parallel with the reticulocyte rise. Serum alkaline phosphatase in A.B. was 2.7 to 2.8 Bodansky units per 100 cc before and after treatment except for a value of 3.2 units on the day when serum and urine phosphorus were greatest. An increase in urinary uric acid-creatinine ratio accompanied reticulocytosis. Similar changes occurring after vit. B_{12} in pernicious anemia patients were interpreted by us as being due to increased utilization of phosphate accompanying a profound stimulation of nucleoprotein metabolism(8).

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‡ This patient received 15 μg on 8th and 9th days.

Summary. The thiocyanate analog of vit. B_{12} (thiocyanato-cobalamin) has been shown to be fully active hematologically in pernicious anemia. In two patients the early biochemical and hematologic responses were the same as observed with same dosages of vit. B_{12}. Further evaluation of the neurological response to this analog is necessary.

Received May 24, 1951. P.S.E.B.M., 1951, v77.

Ergothioneine Depletion in Rabbit Erythrocytes and Its Effect on Methemoglobin Formation and Reversion. (18799)

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Recent studies on methemoglobin (MHb) formation and reduction in nitrite-treated rabbit erythrocytes revealed an acceleration in both the appearance and reversion of MHb in red cells of animals raised on a purified diet(1). Liver paste added to this diet retarded the MHb formation rate but various crude supplements such as liver, yeast, wheat germ, and kale failed to alter the MHb reversion rate. Supplements of ergothioneine, glutathione, and vit. B_{12} have since been tested and it has been found that the ergothioneine retards MHb formation or reduction to a rate characteristic of corpuscles of animals raised on a stock diet. Moreover, the blood ergothioneine level varies inversely with the MHb rates, being low in rabbits on the purified diet and high in those fed the stock diet or treated with ergothioneine.

Procedure. Fifty male New Zealand strain albino rabbits weighing 1600-1800 g were placed on the purified diet designated as No. 672, while 12 animals were put on the stock diet composed of Purina rabbit pellets(1). Diet 672 contained sucrose, casein, cellophane, Wesson oil, O.M. salt mixture and vitamins as described previously. After 5 months the test rabbits were separated into 5 equal groups. Equal blood samples of the individuals in each group were pooled, and the rate of MHb formation was determined in the washed substrate-free erythrocytes following addition of a standard dose of $NaNO_2$. This test and the determination of the rate of reversion of MHb after addition of a standard $NaNO_2$ dose to corpuscles suspended in a medium containing lactate as substrate were carried out according to the procedure already outlined(1). Supplements of vit. B_{12}, ergothioneine and glutathione, were then given three groups (Table I). Three months later the MHb tests were repeated, and in addition the erythrocyte ergothioneine and glutathione content was measured using the most recent Hunter method for ergothioneine(2). In or-

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TABLE I. Effect of Diet on Blood Ergothioneine and the Rate of MHb Formation and Reversion. E designates 6.7 mg % ergothioneine, G–SH 10 mg % glutathione, B_{12} 8.3 γ % vit. B_{12}, L 1% liver paste (Valentine's 1:20 concentrate). Flasks for MHb formation tests contained 2 ml washed corpuscles + 6 ml .85% NaCl + .15 ml 1% $NaNO_2$. Flasks for MHb reversion tests contained 2 ml washed cells + 2 ml M/4 sodium lactate + .3 ml 1% $NaNO_2$.

Duration of exp.	Supplement to diet 672			MHb formation: g % MHb 7 min. after addition of $NaNO_2$			MHb reversion: time in hr for MHb to return to 3 g %			mg ergothioneine/100 ml of erythrocytes*	
(mo.)	5	8†	10†	5	8	10	5	8	10	8	10
Purified diet	0	B_{12}	0	4.8	5.4	4.6	1.83	2.17	1.83	0	0
	0	E	0	5.2	1.8	1.7	1.83	2.67	2.58	23.1	14
	0	G–SH	B_{12}	4.7	5	5.2	1.75	1.75	1.50	0	0
	0	0	L	4.8	5.4	3.8	1.58	1.75	1.75	0	4
	0	0	E	—‡	4.7	3.5		1.83	2.25	0	9.6
Stock diet				.7	1.8	1	2.08	2.58	2.83	7	11

* A faint yellow color equivalent to 1-2 mg % ergothioneine was encountered in purified diet cell filtrates but only characteristic pink color was interpreted as representing ergothioneine.

† Duration indicated by 8 is from 5 mo. through 8 mo., that by 10 is 8 mo. through 10 mo.

‡ Not tested at this time.

der to check the findings, an altered supplemental regime was instituted for a final two months after which the MHb and ergothioneine determinations were repeated.

Results. At the end of the 5-months' induction period the rate of appearance and disappearance of MHb was found to be uniformly rapid for corpuscles of all the experimental groups as compared with the stock diet controls (Table I). After the various supplements to diet 672 had been consumed for 3 months the rate of formation and reversion of MHb became retarded in the cells of the group given ergothioneine, whereas no change occurred in the other groups. Ergothioneine determinations showed a very low or negligible amount in the washed erythrocytes of all the test groups except that which received the ergothioneine supplement. The cells of the latter had a higher level than those of stock diet controls.

The diets tested had no influence on the glutathione level, for the concentration of this substance was comparable in the washed cells of the several experimental groups and the controls. The animals were tested again 2 months after the supplements were altered. Some slowing of MHb formation and reversion was observed together with a corresponding increase in blood ergothioneine on adding this substance to the diet of a group previously unsupplemented. The group from which the ergothioneine supplement was withdrawn revealed little change either in the amount of the compound in the blood or the MHb tests. However, the level decreased to 4 mg % 4 months after the ergothioneine was withdrawn. Addition of liver extract to the diet of a previously untreated group caused the blood ergothioneine to increase. The anomalous results encountered in earlier studies with liver supplements recurred in this test—that is, a slowing of the rate of MHb formation without a corresponding change in the time required for MHb to disappear(1).

Certain incidental observations were made. Ergothioneine depletion was found to have no distinct influence on the hemoglobin concentration, hematocrit value, number of circulating erythrocytes, red cell fragility or oxygen dissociation curve. Following a standard dose of aniline, no difference in the rate of appearance of MHb was noted in the 6 groups of Table I. Washing erythrocytes decreased the ergothioneine content approximately 20% and storage at 4°C caused roughly a 50% decrease in a week.

Discussion. Dietary variation in the blood ergothioneine level of pigs and rats was reported by Eagles and Vars(3), and Potter and

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3. Eagles, B. A., and Vars, H. M., *J. Biol. Chem.,* 1928, v80, 615.

Franke(4), respectively. However, Touster, using the bromine-labile sulfur method(5) as well as the Hunter method of ergothioneine assay, has failed to confirm the latter results. Hunter recently, unlike Eagles and Vars, did not find that maize meal contains an ergothioneine precursor(6). The definite dietary influence on blood ergothioneine reported here, while possibly a species characteristic, is quite likely at least in part also attributable to the fact that the animals were started on the experimental diet at weaning.

Absence of an ergothioneine protective action against oxidation of the hemoglobin could account for the occurrence of an unidentified hemoglobin derivative previously observed in erythrocytes of the purified diet rabbits(7). Likewise, ergothioneine differences may be implicated in the individual and age differences previously observed in MHb reversion(8).

Summary. 1. Erythrocytes of rabbits fed a purified diet are nearly or completely devoid of ergothioneine. 2. The amount of ergothioneine in the red cells of purified diet rabbits increases with a supplement of liver or ergothioneine. 3. MHb formation and reversion rates after $NaNO_2$ are related inversely to the ergothioneine blood levels. 4. The diets used do not influence the blood glutathione

The authors are indebted to Mr. Howard F. Brubach for valuable assistance in aspects of this work.

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Received May 26, 1951. P.S.E.B.M., 1951, v77.

Effect of Diet on Obesity of Yellow Mice in Inbred Lines.* (18800)

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It has been well known for many years that obesity is associated with yellow coat color in mice. That continued inbreeding reduces the obesity is less widely known but has been the experience of several mouse geneticists who have developed inbred lines of this stock. In mice the gene (A^y) for yellow coat color is dominant over the other alleles (A^w, A, a^t, a) at this agouti locus, and the homozygote ($A^y A^y$) is lethal. Consequently yellow mice are always heterozygous for yellow and produce both yellow and non-yellow offspring. By continued inbreeding of a yellow stock (successive brother-sister matings with one or both parents yellow), complete homozygosity at all loci is approached except at the A locus. In this manner yellow and non-yellow littermates are obtained having the same genetic background and differing only in that the yellows have one A^y gene and the non-yellows have none. Danforth(1), by crossing yellows to certain unrelated lines, showed that it was possible to increase the expression of obesity in the F_1 hybrid yellows. This practice of obtaining F_1 obese yellows has been employed in other studies on obesity(2,3). In the experiments to be reported here, however, such hybrid yellows were not used.

Two inbred yellow sublines (one pink-eyed and the other not) have been developed by one of us (H.B.C.). In view of the failure of the yellows of these sublines (and certain ones

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*This investigation was supported by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council; The National Cancer Institute, U. S. Public Health Service; and the Anna Fuller Fund.

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2. Dickerson, E. G., and Gowen, J. W., *Records Gen. Soc. Am.*, 1946, v14, 44.
3. Dickie, M. M., and Woolley, G. W., *J. Hered.*, 19..., v37, 365.

in other laboratories) to become obese under the usual conditions of colony maintenance, the question arose whether dietary manipulations could still bring out a capacity to become obese not shared by the non-yellow littermates.

Methods. Exp. 1. Two inbred yellow sublines were developed from the yellow stock of Jackson Laboratory. One subline is of the genotype $CCA^y/abbDDppss$ and is pink-eyed yellow and café-au-lait (non-yellow). The other subline is of the genotype $CCA^y/aBBDDPPss$ and is normal-eyed yellow and black (non-yellow). Both sublines are white-spotted. Since there is no difference in weight between these sublines, they will be considered together as the yellow strain. Yellow and non-yellow littermates were weaned between 21 and 28 days of age. They were maintained in plastic cages, one litter per cage, and fed a commercial stock ration. Food and water were supplied *ad libitum*.

Exp. 2. Yellow and non-yellow littermates of the sublines used in Exp. 1 were weaned when 21 days of age. At this time they were placed in individual screen-bottom metal cages and fed diet No. 226 or a commercial stock ration. Diet No. 226 was composed of casein 30%, sucrose 17.5%, corn oil 5%, hydrogenated cottonseed oil 42.5%, and salt mixture A(4) 5%. Fat- and water-soluble vitamins were added to this mixture in the amounts previously reported(4). Food and water were supplied *ad libitum*. The animals were weighed every 3 days until 51 days of age. Thereafter they were weighed once each week. They were sacrificed at 6 months of age and carcass fat determined by the method described by Feyder(5). A total of 72 mice were used in these 2 experiments.

Results. Exp. 1. At 6 months of age yellow mice were only slightly heavier on the average than their non-yellow littermates. At one year of age only one yellow male reached a weight of 50 g, all other animals weighing less than 41 g.

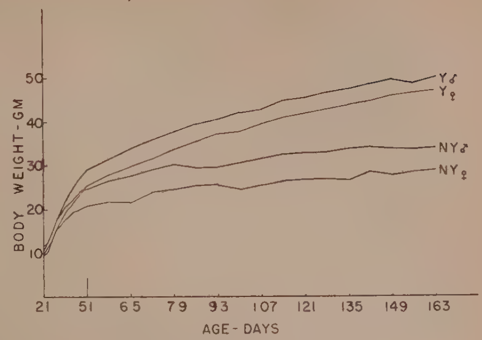

FIG. 1. Growth Curves of Yellow and Non-yellow Mice Fed Diet #226.

Exp. 2. The weight gains of yellow and non-yellow males and females fed diet No. 226 are shown in Fig. 1. Only one yellow male gained less weight during the experimental period than did the heaviest of the non-yellow males. Table I summarizes the fat content of the several experimental groups fed the highly purified diet. At the time of autopsy all yellow mice were found to have fatty livers, and two of them had liver tumors of moderate size. No comparable difference in weight gains was noted between yellow mice and non-yellow littermates when raised on the commercial stock ration.

Discussion. The experiments reported here confirm the observation that yellow mice maintained under the usual laboratory conditions (housed either singly or in groups) did not become obese although they attain a body weight slightly greater than that of their non-yellow littermates. It is clear, however, that mice with the yellow gene, A^ya, still retain their capacity to become obese when fed suitable diets. The littermates homozygous for the non-yellow allele, aa, do not possess this capacity as far as we have been able to determine. The induction of obesity in yellow

TABLE I. Fat Content of Yellow and Non-Yellow Mice Fed Diet #226 until 6 Months of Age.

	Total body wt, g	Total fat, g	Fat-free wt, g	Fat content, %
Yellow males	50.7	17.2	33.5	33
” females	49	17.7	31.3	35.9
Non-yellow males	34	5.6	28.4	16.3
” females	27.7	4.2	23.5	14.6

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4. Fenton, P. F., and Carr, C. J., *J. Nutrition*, 1951, v43, 441.

5. Feyder, S., *J. Nutrition*, 1935, v9, 457.

mice by the feeding of a diet of the "synthetic" type might be attributed to the greater nutritional adequacy of the synthetic diet as compared with the commercial pellet, or to the greater fat content of diet No. 226. Further experiments are needed to answer this question. Fatty livers such as shown by the yellow mice fed diet No. 226 have been reported in mice of the A and C_3H strains by Fenton and Mershon(6).

The occurrence of two liver tumors seems worthy of mention although no data are available on the incidence of liver tumors in these yellow sublines. The existence of such tumors in animals only 6 months of age merits further attention.

Summary and conclusions. 1. The observation has been confirmed that yellow mice on continued inbreeding no longer become obese if maintained under the usual laboratory conditions. 2. Feeding a diet of the "synthetic" type made it possible to induce obesity in the yellow mice but not in their non-yellow littermates. 3. The obese yellow mice were observed to have fatty livers. Two of these animals were found to have liver tumors.

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6. Fenton, P. F., and Mershon, J. S., *Fed. Proc.*, 1951, v10, 382.

Received May 28, 1951. P.S.E.B.M., 1951, v77.

Effect of Nitro-Compounds and Aldehyde Semicarbazones on Virus of Primary Atypical Pneumonia.* (18801)

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Since the atypical pneumonia virus is intermediate in size between the agents of the psittacosis-LGV group and the so-called "true" viruses like influenza it may occupy in relation to chemical inhibition a strategic position one step down the scale from the "bacteria-like" viruses and rickettsiae for which many inhibitory substances have already been found. For this reason further exploration of the relation of chemical structure of certain substances to antiviral activity has been undertaken with this agent.

In a previous publication(1) it was reported that chloramphenicol had little effect on the pulmonary lesions produced in cotton rats by intranasal inoculation of the atypical pneumonia virus, but more recent studies to be reported here indicate activity in chick embryos. Because of the similarity of action of other p-nitro-benzene and 5-nitrofuraldehyde derivatives particularly those containing a semicarbazone group to that of chloramphenicol against respiratory infection in mice with viruses of the psittacosis-LGV group(2) it was considered of interest to study the effect of these substances against the atypical pneumonia virus. Experiments were also done with para-amino-benzaldehyde thiosemicarbazone and its acetyl derivative which have recently been reported to show slight inhibitory activity against vaccinia virus in mice and chick embryos(3).

Materials and methods. The methods of performing the experiments in chick embryos and cotton rats were essentially the same as those described(1). Chick embryos inoculated by the amniotic route with virus received the test substances in the yolk sac. Drugs used for the chick enbryos were sterilized by heat or filtration but for intraperitoneal injection of cotton rats the semicarbazones and thio-semicarbazones were

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* This work was supported in part by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and the Eugene Higgins Trust.

1. Eaton, M. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 24.

2. Eaton, M. D., Huang, C., and Levenson, C. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 501.

3. Hamre, D., Bernstein, J., and Donovick, R., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 275.

TABLE I. Effect of Chloramphenicol and 5-Nitro-Furaldehyde Semicarbazone Derivatives on Growth of Atypical Pneumonia Virus in Chick Embryos.

				Treated eggs		Control eggs	
Exp. No.	Substance	Dosage mg/egg and time interval	Atypical pneumonia strain	No. in pool	Result of passage to C.R.*	No. in pool	Result of passage to C.R.*
1	Chloram.	5 mg 1 hr after	De	5	1/6†	8	4/6
2			”	4	2/8†	6	4/6
3			”	5	0/6	5	5/6
4			Mac	8	0/14	9	7/13
5	NF II‡	.5 mg 2×§	”	13	0/10	6	5/12
6		.5 ”	De	16	3/10	12	6/9
7	NF V‡	1 ”	Mac	8	0/13	7	6/12
8		1 ”	”	6	9/16	10	8/15

* No. of animals with pulmonary lesions over total tested.

† Very small pulmonary lesions.

‡ NF II is 5-nitro-2-furaldehyde 2-β hydroxyethyl semicarbazone. NF V is 5-nitro-2-furaldehyde 4-(Diethylaminopropyl) semicarbazone.

§ Two doses at 2 and 48 hr after virus.

ground and suspended in saline without heating. Dosages of drug up to one third or one half the LD_{50} were used in these experiments. Amounts given intraperitoneally to the cotton rats were recorded as milligrams per 100 of body weight. The system of recording pulmonary lesions and calculating lesion scores was the same as that used before. Lesions involving ½ to ¼ of the total volume of lung tissue were recorded as 2, those involving ¼ to ⅛ of the lung as 1, those involving less than ⅛ of the lung as ½. Lesions less than 1 mm in diameter were recorded as ± and may be considered to have doubtful significance.

Results in chick embryos with chloramphenicol and derivatives of 5-nitro-2-furaldehyde semicarbazone. In embryos treated with a single dose of 5 mg of chloramphenicol 1 hour after infection multiplication of virus was apparently inhibited since only 3 of the 34 cotton rats inoculated intranasally from treated embryos developed very small pulmonary lesions while 20 of the 31 animals receiving control material had small or large patches of pneumonia. These results are presented in Table I (experiments 1-4). Since the virus grown in chick embryos infects cotton rats only at dilutions of 10^{-1} or 10^{-2} the reduction of the amount of virus in the treated embryos was 10-fold or more. With the 2 nitrofuraldehyde compounds given in 2 doses 2 and 48 hours after the virus the effects were somewhat irregular. The method could not be expected to detect individual variation between eggs since material from the chick embryos in each experiment is pooled. In Experiment 6 where some virus was detected in the treated group, 16 eggs had been pooled (the largest group in the series) thus giving a greater chance of including one positive embryo.

Results in cotton rats with nitro- or amino cyclic aldehyde derivatives. Two experiments were done in cotton rats with 5-nitro-2-furaldehyde-4-(diethylaminopropyl) semicarbazone (NF V) and one with the 2-β-hydroxyethyl semicarbazone (NF II). As will be seen in Table II both compounds at a dosage of 8 to 10 mg/100 significantly reduced the number of animals with lung lesions, but with a dose of 6.5 mg/100 of NF V (Experiment 10) the results were less favorable. P-nitrobenzaldehyde semicarbazone (NB I Experiments 10 and 11) was included in these studies for comparison with the nitrofuraldehyde semicarbazones. The results appeared to be similar to those with the nitro-furaldehyde semicarbazone. Experiments with p-nitrobenzaldehyde thiosemicarbazone and the corresponding p-amino benzaldehyde derivative at the maximum tolerated dosage gave an apparent reduction of the pulmonary lesions but the results were not as clear cut as with the semicarbazone derivatives.

The acetyl derivative of p-aminobenzaldehyde thiosemicarbazone (AB II) was found to be less toxic for cotton rats than the other substances in this series. When this com-

TABLE II. Direct Treatment of Experimental Pneumonia in Cotton Rats.

Exp. No.	Drug*	Daily dosage mg/100 g body wt	Started at, hr	No. of animals with pulmonary lesions of 2	1	½	±	0	Avg L.S.†	Infection ratio
9	NFV	10	24	0	0	1	2	9	1.7	1/12
	0			1	4	1	2	3	12.7	6/11
10	NFV	6.5	1	0	1	1	2	4	5	2/8
	NBI	6.5	1	0	0	2	0	4	3.3	2/6
	0			1	1	4	1	1	12.5	6/8
11	NFII	8	1	0	0	1	0	6	1.4	1/7
	NBI	10	1	0	0	1	3	5	2.8	1/9
	ABI	6	1	0	0	1	1	3	3	1/5
	0			2	4	0	1	2	19	6/9
12	NBII	8	1	0	1	1	3	6	4.1	2/11
	ABI	8	1	0	1	1	1	4	5	2/7
	0			0	3	4	1	3	9.5	7/11
13	ABII	20	1	0	0	0	0	8	0	0/8
	,,	10	1	0	1	1	1	5	4.4	2/8
	,,	5	1	0	1	0	0	7	2.5	1/8
	0			0	5	3	2	5	8.8	8/15

* NBI = p-nitro-benzaldehyde semicarbazone; NBII = p-nitro-benzaldehyde thiosemicarbazone; ABI = p-aminobenzaldehyde thiosemicarbazone; ABII = p-acetylamino benzaldehyde thiosemicarbazone.

† Lesion scores represent % of pulmonary consolidation—see section on Methods and Materials.

pound was given in daily doses of 20 mg per 100 g body weight, complete suppression of the pulmonary lesions was observed in one Experiment (No. 13) but when the dosage was lowered to that used with the p-nitro- or p-amino-benzaldehyde thiosemicarbazone the results were similar to those obtained with the latter two compounds.

Discussion. Despite the failure of chloramphenicol to affect materially the course of the infection with atypical pneumonia virus in cotton rats(1) this compound was found in the present study to inhibit the growth of the virus in chick embryos. Extension of these observations to other nitro compounds revealed that two derivatives of 5-nitro-2-furaldehyde semicarbazone in contrast to chloramphenicol produced only irregular inhibition of the growth of the virus in chick embryos but did have a rather marked effect in reducing the development of pulmonary lesions in cotton rats when used at the maximum tolerated dosage. Two compounds of related structure, which have not been previously tested, p-nitrobenzaldehyde semicarbazone and thiosemicarbazone also showed some inhibitory effect in cotton rats.

The observation that p-acetylaminobenzaldehyde thiosemicarbazone when used in large doses completely suppressed the pulmonary lesions in cotton rats indicates that the nitro group is not essential to this effect. All of the compounds active in cotton rats contained the semicarbazone or thiosemicarbazone structure while chloramphenicol which had little or no activity in these animals has a different side chain. Results in some of the earlier studies with the psittacosis-LGV group of viruses were analogous to the recent findings with atypical pneumonia virus. Against the psittacosis-LGV group of viruses chloramphenicol showed greater activity in chick embryos than against experimental pneumonia in mice and this was true of other chemotherapeutic agents such as penicillin, nitro-acridine and p-nitro-benzamide(2). Nitrofuraldehyde semicarbazone showed greater activity against pulmonary infections with lymphogranuloma venereum and meningopneumonitis viruses in mice than did chloramphenicol while in chick embryos the reverse was true. These observations suggest that the semicarbazone or thiosemicarbazone group may confer some special activity against pulmonary infections and the possibility of a general physiological effect in the animal as a factor in the mode of action of these compounds should be considered.

Studies to measure the degree of inhibition by various substances of viral growth in lungs in comparison with the amount of pulmonary consolidation are being carried on.

None of the semicarbazones or thiosemicarbazones used in these studies appears to have as favorable a chemotherapeutic index as aureomycin or chloromycetin so that there is nothing to indicate their clinical usefulness in the treatment of atypical pneumonia. The observed relation of chemical structure to activity against certain viruses of large particle size including recent reports of slight activity against vaccinia in chick embryos and mice (3,4) seems of interest from the fundamental point of view and as a suggestion for further studies with other viruses in the same range of particle size.

Summary. 1. Chloramphenicol inhibited the growth of primary atypical pneumonia virus in chick embryos when injected into the yolk sac as a single dose of 5 mg one hour after amniotic inoculation of virus. Less definite inhibition of the growth of this virus in chick embryos was observed with two derivatives of 5-nitro-2-furaldehyde semicarbazone. 2. Cotton rats infected intranasally with the virus of atypical pneumonia were treated by intraperitoneal injection of several different cyclic aldehyde semicarbazone and thiosemicarbazone derivatives. Most marked suppression of the pulmonary lesions was obtained with substituted 5-nitro-2-furaldehyde semicarbazones, p-nitrobenzaldehyde semicarbazone, and p-acetylaminobenzaldehyde thiosemicarbazone.

The authors wish to acknowledge with thanks the generous supply of compounds furnished by Dr. A. F. Langlykke of the Research and Development Laboratories of E. R. Squibb and Sons, and by Dr. Mary F. Paul of the Eaton Laboratories, Inc.

Received May 31, 1951. P.S.E.B.M., 1951, v77.

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An Epizootic of Unknown Etiology in Guinea Pigs.* (18802)

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Several workers in diagnostic and research laboratories have observed extraneous diseases among their experimental guinea pigs. Römer (1) discovered a sporadic disease in guinea pigs which he termed infectious paralysis. The virus of this disease could be passed indefinitely by brain to brain transfer. Jackson(2) described intracellular parasites in the submaxillary glands of guinea pigs, but these were shown by Cole and Kuttner(3) to be inclusion bodies. Later Kuttner(4) found these inclusions were due to a specific salivary gland virus, and succeeded in passing the virus for 3 passages to susceptible young guinea pigs. Rosenbusch and Lucas(5) encountered a salivary gland virus of pronounced virulence while engaged in a genetics experiment; the virus was peculiar in that it was pathogenic for guinea pigs by various routes of inoculation. While conducting a nutritional experiment, Pappenheimer and Slanetz(6) were confronted with a spontaneous visceral disease in their guinea pigs. The findings were suggestive of a viral etiology, although attempted

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* This investigation was aided by the Central Scientific Fund, College of Medicine, State University of Iowa.

1. Römer, *Zbl. Bakt.*, 1911, v50, 30.
2. Jackson, L., *J. Infect. Dis.*, 1920, v26, 347.
3. Cole, R., and Kuttner, A. G., *J. Exp. Med.*, 1926, v44, 855.
4. Kuttner, A. G., *J. Exp. Med.*, 1927, v46, 935.
5. Rosenbusch, C. T., and Lucas, A. M., *Am. J. Path.*, 1939, v15, 303.
6. Pappenheimer, A. M., and Slanetz, C. A., *J. Exp. Med.*, 1942, v76, 299.

passage of the agent in question yielded negative results. Van Rooyen and Rhodes(7) have described lymphocytic choriomeningitis infections in guinea pigs, and mention the possibility of wild mice being involved in the transmission of the agent. It appears likely that wild mice, or even stock experimental mice, harboring the latent infection may possibly infect susceptible guinea pigs under proper conditions. Markham(8) observed that stock guinea pigs were sometimes spontaneously infected with toxoplasmosis, and serial passage in susceptible animals was easily accomplished by brain to brain transfer.

It is the purpose of this report to describe an epizootic of 11 months duration that appeared among the guinea pigs used for tuberculosis testing by the Diagnostic Laboratory of the Bacteriology Department of the State University of Iowa in the latter part of July, 1949. This disease often interrupted the 6 weeks observation period 2 to 4 weeks prior to gross and microscopic examination of the animals for evidence of tuberculosis. All cases of the disease followed a very definite symptomatology. At the onset of symptoms the animals became unresponsive and sluggish, their coats became very shaggy and the hair shed easily, and they had a marked loss of appetite. The loss of appetite was followed by a decided loss of weight and emaciation. There was a transient increase in water consumption at this point of the disease, but soon the animals refused to drink. About this same time the guinea pigs were observed to be quite damp around the area of their chins, mouths, and ventral surface of their necks. The continual dampness of the affected pigs was perhaps the most outstanding symptom associated with the disease. In more severe cases the dampness extended posteriorly over the entire ventral surface of the abdomen. The nature of this dampness was most likely attributable to a constant nausea, accompanied by frequent vomiting, and the vomitus possessed a very acrid odor. Death of a guinea pig with such symptoms usually occurred within 6 days. When autopsied, the most significant finding was the almost complete loss of subcutaneous fat. Most of the animals showed a slight amount of hemorrhage in the meninges of the brain, and about 5% showed some lung consolidation due to a secondary pneumonia from which an alpha-prime hemolytic streptococcus was isolated.

Materials and methods. Guinea pigs used in these studies were obtained from our regular market source, and also from a different market source, and all were about 6 weeks old when inoculated. Four-week-old Swiss white mice were used from our own disease-free inbred stock. Fertile eggs were obtained from an accredited local hatchery, and the embryos were 10-11 days old when inoculated. Attempts were made to pass the etiological agent from infected to healthy guinea pigs by using various routes of inoculation, and by employing several combinations of inocula from infected animals. On one occasion, a single blind passage to healthy experimental animals from nine guinea pigs, which had been inoculated with a composite organ extract from infected animals failing to develop characteristic symptoms, was attempted. Throughout these studies complete autopsies were performed on diseased guinea pigs, and tissues were submitted to the Department of Pathology for their examination.† The possibility that we were dealing with some type of latent infection led to the inoculation of several experimental guinea pigs with materials other than organ extracts from diseased animals. The materials chosen were sterile human serum, ether extracted human serum, the lipid residue from the ether extraction, sterile milk, normal horse serum, viable B.C.G. with and without mucin, inactivated *Mycobacterium tuberculosis* with and without mucin, and gastric mucin of porcine origin.

Seven experimental animals from our regular market source and three from another source were held as uninoculated controls in the same room as the inoculated ones. Some

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7. Van Rooyen, C. E., and Rhodes, A. J. Virus Diseases of Man, 1051-1060. Thomas Nelson and Sons, New York City, 1948.

8. Markham, F. S., *Am. J. Hyg.*, 1937, v26, 193.

† Appreciation and thanks are extended to Dr. Jack M. Layton of the Department of Pathology for histological examinations during these studies.

of the guinea pigs from each market source were allowed intimate contact with diseased animals, whereas others were held in separate cages as room contacts. To determine whether adult guinea pigs from our regular market source had developed an active immunity to the etiological agent in question, 10 experimental guinea pigs were inoculated intraabdominally with 1 ml of pooled adult guinea pig sera and held for observation. Attempts to transfer the causative agent to mice were made by inoculating 24 male and 32 female Swiss mice with a bacteriologically sterile composite organ extract of lung, brain, liver, spleen and spinal cord from infected experimental animals. Each mouse received 0.03 ml intracranially and intranasally and 0.3 ml intraabdominally. Using 0.2 ml of the same inoculum, 12 ten-day-old chick embryos were inoculated into the allantoic sac and onto the chorio-allantoic membrane. Penicillin and streptomycin were not added to the inoculum. The embryos were incubated at 37°C for 48 hours, after which the chorio-allantoic fluids and membranes were harvested. The chorio-allantoic fluid and supernatant fluid from the chorio-allantoic membranes from the first egg passage were subsequently inoculated into another 12 embryos as performed in the first egg passage. Thirty-six Swiss mice were inoculated with material from the harvests from both egg passages, 0.03 ml intracranially and 0.30 ml intranasally. Nine infected experimental animals, chosen from random, were treated with four antibiotics simultaneously. Each animal received daily intramuscular injections of 0.01 g of dihydrostreptomycin, 40,000 units of S.R. penicillin, 2.5 mg of chloramphenicol, and 2.5 mg of aureomycin. Four animals were treated 4 days, and 5 for 5 days.

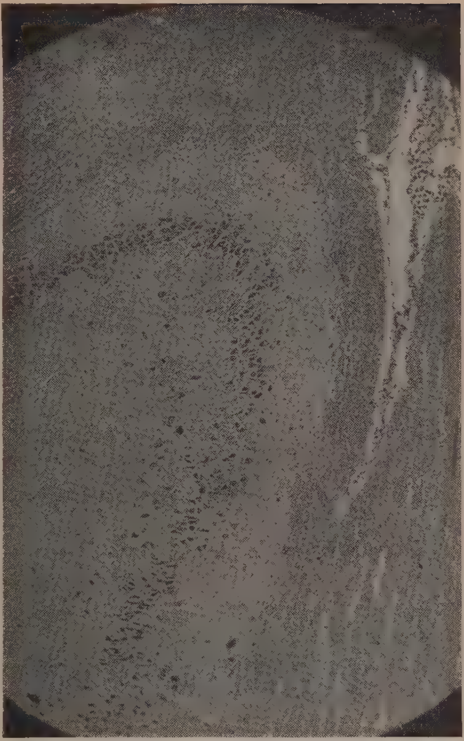
FIG. 1. Section Through Hippocampus of Diseased Guinea Pig, Low Power.

TABLE I. Results of Attempted Passage in Guinea Pigs.

No. of guinea pigs	Inoculum	Route of inoculation*	Avg days to symptoms	Avg days to death
6	brain + spinal-cord	I.C.	59	64
8	” ” ”	I.A.	42.5	48.5
5	brain, spleen, liver, kidney, adrenal glands	I.A.	—	—
1	liver, spleen	I.C.	62	66
1	” ” , axillary node	I.C.	51	54
1	salivary gland	I.C.	50	53
Totals 22			44.1 avg	47.7 avg

* I.C.—intracranial; I.A.—intraabdominal.

Results. Attempted passage from infected animals to 22 healthy experimental guinea pigs, by using the routes and various inocula shown in Table I, resulted in 17 infections. The average number of days to symptoms from date of inoculation was 44.1 and from symptoms to death 47.7 days. The one blind passage from 9 inoculated but uninfected experimental animals to healthy guinea pigs resulted in no infections.

The inoculation of 10 experimental animals in an attempt to provoke the disease with sterile milk, human serum, and other materials mentioned under methods, resulted in 8 of the 10 becoming infected in an average time of 48.5 days from date of inoculation. At this point attention is called to the close similarity between the apparent incubation period in this experiment and the one obtained in attempted passage from infected to healthy guinea pigs.

Among the uninoculated control guinea pigs from two market sources, 8 of the 10 animals developed characteristic symptoms of the disease and succumbed. The average incubation period for those intimately exposed in both groups was 72 days, and for those held in separate cages as room contacts 85 days. No differences in susceptibility of the guinea pigs from the two sources were apparent.

The attempt to protect young healthy guinea pigs by inoculating them with pooled adult guinea pig sera resulted in failure. It was thus apparent that none of the 10 young experimental animals received passive protection from adult guinea pigs from the same stock. Attempts to transfer the etiological agent from infected guinea pigs to mice, and from infected guinea pigs to chick embryos for 2 passages and then to mice, all gave negative results. Mice failed to develop symptoms of any kind after a 6 weeks' observation period, and chick embryos failed to develop lesions and showed no toxic manifestations either on the first or second passage. Guinea pigs exhibiting typical symptoms failed to respond to treatment using four antibiotics simultaneously.

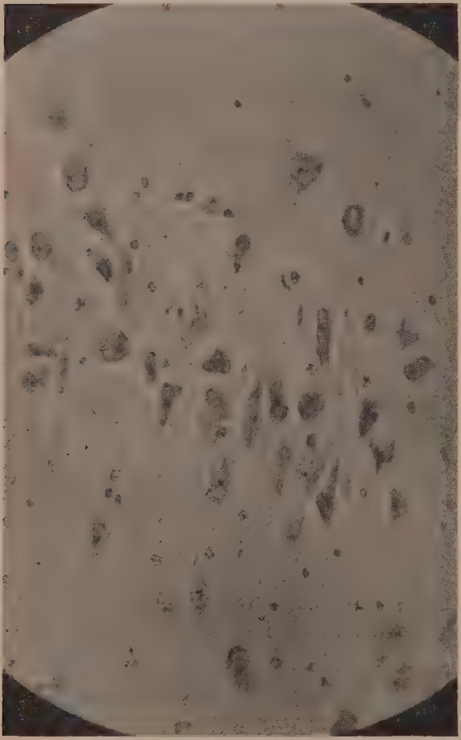

FIG. 2. Section Through Hippocampus of Diseased Guinea Pig, High Power, Showing the Degenerative Changes in the Neurones.

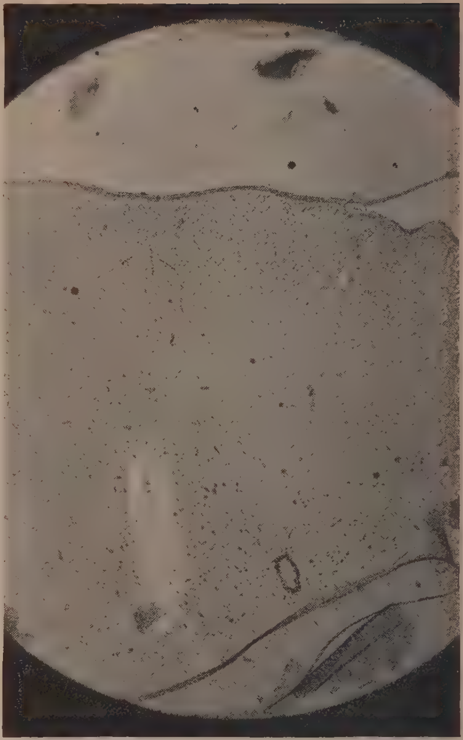

FIG. 3. Cross-Section of the Cervical Spinal Cord from a Diseased Guinea Pig, Low Power, Showing Demyelination in the Postero-Lateral Columns.

Histological sections were examined from the following tissues: Heart, lungs, thymus, lymph nodes, pancreas, skeletal muscle, gastrointestinal tract, skin, kidney, adrenal, liver, salivary glands, testicle, central nervous system, and cranium. The only suggestive anatomic abnormalities encountered were in the central nervous system. Degenerative changes in the neurones of the hippocampal gyrus were constant in all of the sections from diseased animals (Fig. 1 and 2). The exact significance of the changes is difficult to evaluate. Demyelination of the postero-lateral columns of the spinal cord and patchy degeneration of neurones of the anterior horns were less constant features (Fig. 3).

Summary. Because of the inconsistency obtained in attempted passage of the etiological agent to susceptible guinea pigs, it is postulated that the etiological agent was not being passed at all. It appeared that many of the inoculations merely served to prematurely hasten an unknown etiological agent on its way, culminating in the disease pattern and the epizootic which has been described.

Conclusions. An epizootic of unknown etiology has been observed in guinea pigs. The disease was highly fatal. The symptoms and pathology were unlike any previously described disease of guinea pigs.

Received June 1, 1951. P.S.E.B.M., 1951, v77.

A Still for the Continuous Production of Glass-Distilled Water.* (18803)

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It is frequently necessary in biological work to have a continuous supply of glass-distilled water available. The apparatus described here has proven to be entirely satisfactory for this purpose. It has been in operation in our laboratories for over a year, 24 hours a day, without a serious breakdown. During this time it has produced on the average 70 liters of glass-distilled water per day. The design is flexible and may be adapted to larger or smaller needs. The only manual labor involved in operating the still consists of filling the storage flasks and emptying the receiver flasks.

The still is shown in Fig. 1. A few features of the construction need to be emphasized. For proper operation the upper storage bottle should be tightly sealed. The condenser D on the siphon tube will condense all steam formed in this tube, thereby preventing breaking of the siphon. There should be a rapid flow of cooling water through both of the condensers. The Allihn condenser L was found to be the most efficient condenser for the purpose. The close clearance between the bulbs and the jacket produces high cooling-water velocities. The use of a Graham condenser is to be avoided due to the large back pressure produced. The vent tube on the receiver is ca 3 cm below the outlet of the condenser and leads into a drain. This is to prevent the closing of the system when the receiver is full. The outside diameter of the tubes used for the siphon and the vent system should be 8 mm. The vent on the receiver flask can either drain into the sink, or may lead to a second storage bottle. In order to prevent contamination with grease, none of the joints is greased. Glass beads or boiling stones are not required because of the even heat furnished by the heating mantle. Phosphoric acid or permanganate can be added to the boiling flask, should this be desirable. The operation of the still should be interrupted occasionally for cleaning the distilling flask. The still can be adapted to larger capacities by increasing the size of the reservoirs and the boiling flask.

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* Contribution No. 467 of the Department of Zoology, Indiana University. Supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research, The National Institute of Health of the U. S. Public Health Service, The Rockefeller Foundation (grant for research on *Paramecium* genetics), and Indiana University.

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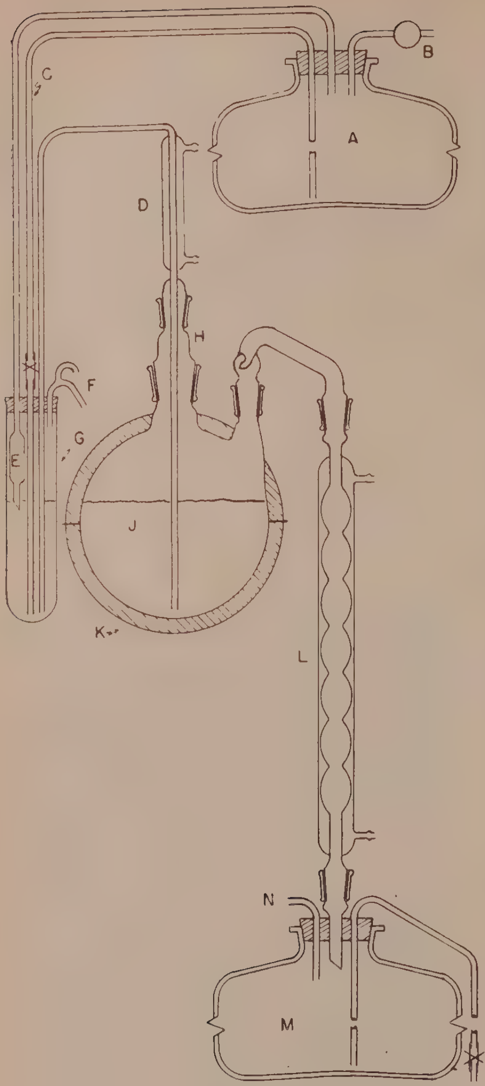

FIG. 1. All-Glass Still for Distilled Water. A: upper reservoir (12 gal); B: distilled water valve and line; C: siphon tube between upper reservoir and leveling bulb; D: condenser for siphon tube from the leveling device to the distilling flask; E: leveling bulb (should have slanted end to prevent ''hang-up'' in the bulb); F: overflow vent tube from leveling device; G: leveling tube; H: 55/50 to 24/40; I: adapter; J: distilling flask (12 l); K: Glascol heating mantle; L: condenser; M: receiving reservoir (12 gal); N: vent and overflow for receiving reservoir.

For the operation of the still, the following manipulations are required: (a) filling of the upper reservoir A. Close the pinch clamp on the siphon tube just above the leveling device. Open valve B to fill the reservoir A. When full, close the tap and open the pinch clamp. The boiling flask will now fill up to the level of the bottom of the leveling bulb E; (b) start the condensers; (c) start heating with the bottom mantle only. When the distillate starts coming over the top mantle can be turned on. (There is danger of cracking the distilling flask, if the top mantle is connected before the distillation has started.)

The still will now operate without supervision. Our still is so adjusted that it will distill about 38 liters of water in 15 hours. Consequently, if at the end of the working day the upper reservoir is filled and the receiver is drained, the latter one will be full at the start of the next day.

Received June 5, 1951. P.S.E.B.M., 1951, v77.

Effect of Small Amounts of Aureomycin and Terramycin on Virus of Feline Pneumonitis. (18804)

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(Introduced by F. M. Hanger.)
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The phenomenon of natural and acquired resistance of microorganisms to antibiotic agents is one of great theoretical as well as practical interest. First noted in connection with penicillin, acquired resistance of staphylococci was regarded as an adaptation by the organism to an environment containing sublethal concentrations of the drug. Later this was shown to occur in a step-wise fashion, and in all likelihood to depend on a selection of mutants occurring spontaneously in large bacterial populations, and involving multiple genetic steps. This is spoken of as the "penicillin type" of acquired resistance. Generally speaking it often tends to be of a low order of magnitude, and is not of great clinical significance. The second, or "streptomycin type" of acquired resistance is much more abrupt and may result in the emergence of a completely resistant form in consequence of a single genetic step. It is this "streptomycin type" of acquired resistance which is of great clinical importance, and has limited the therapeutic usefulness of that particular antibiotic. Parenthetically it may be remarked that the phenomenon of natural resistance of many strains of staphylococci and a few other microorganisms to penicillin associated with the production of the enzyme "penicillinase" has not been noted in connection with other antibiotics. With the development of new antibiotics, it has naturally been of interest to study the phenomenon of acquisition of bacterial resistance in each, and reports have already appeared in the literature concerning chloramphenicol, aureomycin and terramycin. These in general indicate, at least in connection with the latter two, that resistance when developed, is of a low order of magnitude, that it is of the "penicillin" rather than the "streptomycin" type, and unlikely to prove of much clinical significance. It may be remarked that in usual therapeutic concentrations, the effect of these new antibiotics appears to be mainly bacteriostatic rather than bactericidal. In addition to their antibacterial properties, these new agents also possess significant action against infections due to rickettsiae and large filterable viruses of the psittacosis-lymphogranuloma group. Here again, their action would appear to be rickettsiastatic and virustatic, rather than a killing one. In view, therefore, of the already reported appearance of bacterial resistance to these agents, it was deemed of considerable interest to inquire whether an analogous phenomenon could be established in connection with a virus. For this reason we have been led to perform the following experiments with aureomycin and terramycin, using the virus of feline pneumonitis as our viral agent, in an effort to determine whether acquired virus resistance to an antibiotic could be demonstrated.

Wong and Cox(1) first reported suppression of growth in embryonated eggs treated with aureomycin of viruses of the psittacosis-lymphogranuloma group, including feline pneumonitis. We(2) have studied both aureomycin and terramycin in the treatment of infection with feline pneumonitis following intranasal inoculation of mice, and it was observed that both agents were highly effective in preventing gross pulmonary lesions if treatment was begun early, and in reversing them when treatment was delayed. The experiments to be described below, wherein we endeavored to produce drug-resistant strains of feline pneumonitis virus, were performed by two methods—the passaging of virus serially in embryonated eggs with varying amounts of the anti-

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* Traveling Fellow from Middlesex Hospital, London.

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2. Kneeland, Y., Jr., and Price, K. M., *J. Immunol.*, 1950, v65, 653.

TABLE I. Intranasal Infection of Mice with 0.02 cc of Feline Pneumonitis Virus, 10^{-2} Dilution.

Control mice	3+ *		1+		0		3+				
(untreated)	3+		2+		0		4+				
	3+	pooled	3+		1+		4+				
	2+	→	3+	pooled	1+		4+				
	0		3+	→	2+		4+				
			3+		3+		4+				
					3+	pooled					
					3+	→					
Aureomycin-	2+		±		0		0				
treated mice	1+		±		0		0				
	±	pooled	1+	pooled	0		0				
	3+	→	1+	→	1+		0				
	2+		0		1+	pooled	0				
	±		0		±	→	0				
							0				
Terramycin-	2+		0		0		1+		0		0
treated mice	1+		±		1+		1+		±		0
	2+	pooled	2+	pooled	1+		1+		1+		0
	2+	→	2+	→	2+	pooled	2+		1+		0
	0		2+		2+	→	2+	pooled	1+	pooled	0
	0		2+		2+		2+	→	1+	→	0

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* Each symbol represents one mouse. The following code is employed: 4+ = nearly total pulmonary consolidation; 3+ = extensive consolidation; 2+ = approximately one-half consolidation, or innumerable focal lesions; 1+ = small areas of consolidation, or widely scattered focal lesions; ± = minimal gross pathologic change.

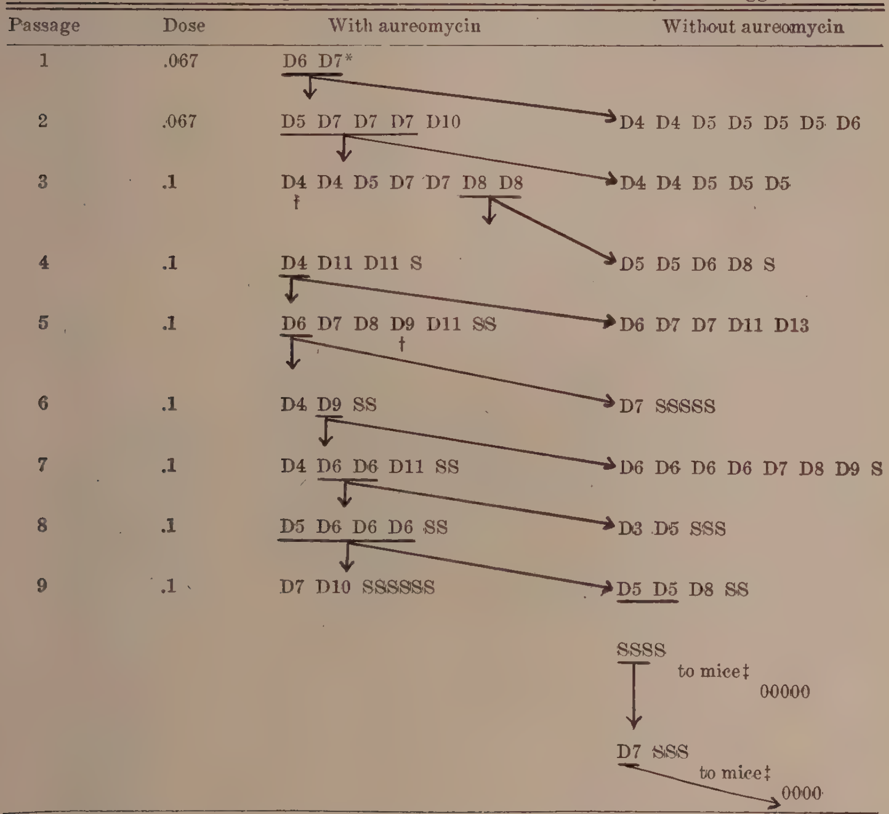
biotic agent to be tested, and the serial passage of virus intranasally in mice with subcurative doses of the antibiotic.

The Baker strain of feline pneumonitis virus employed was originally obtained through the courtesy of Dr. Geoffrey Rake in the form of a lyophilized yolk sac membrane. After 2 further egg passages and 3 more mouse passages, it was used to initiate the mouse experiments in a 10^{-2} lung suspension made up in nutrient broth. The 2 antibiotics studied, aureomycin (Lederle) and terramycin (Pfizer) were administered by subcutaneous inoculation in the form of the hydrochloride dissolved in nutrient broth. Following intranasal inoculation of 0.02 cc of virus, all mice were left 3 days untreated. Thereafter the experimental groups were treated for 4 days with a daily injection of 0.25 mg of antibiotic a dosage which appeared insufficiently large to cause complete regression of the lung lesions, although it had some effect. On the seventh day all mice were sacrificed, including controls, and the lungs passaged to fresh groups of mice. Table I gives the results of these experiments.

It can be seen from the table that the virus in the untreated mice appeared to increase in virulence with repeated passage, so that in the fourth 5 out of 6 mice showed nearly total pulmonary consolidation. By contrast, in the mice treated with 0.25 mg of aureomycin daily for four days beginning on the third day after intranasal inoculation, pulmonary lesions were less conspicuous, tended to diminish, and then completely disappeared in the fourth passage. A similar phenomenon occurred with terramycin administered in the same dosage, except that the effect of the antibiotic was less marked in the early passages, and complete disappearance of virus activity did not occur until the sixth passage. Under the conditions of this experiment, therefore, no evidence of the emergence of a resistant mutant was obtained. In fact the reverse occurred, for the virus, after apparently maintaining itself with some difficulty in an unfavorable environment, proceeded to die out.

After our failure to demonstrate the phenomenon of acquired virus resistance in mice, we undertook to see whether it could be produced when virus was cultivated in embryonated eggs with varying concentrations of aureomycin. In these experiments the virus was mixed with antibiotic and introduced by yolk sac inoculation in a 10^{-3} dilution of in-

TABLE II. Passage of Feline Pneumonitis Virus in Embryonated Eggs.



Passage	Dose	With aureomycin	Without aureomycin
1	.067	D6 D7*	
2	.067	D5 D7 D7 D7 D10	D4 D4 D5 D5 D5 D5 D6
3	.1	D4 D4 D5 D7 D7 D8 D8 †	D4 D4 D5 D5 D5
4	.1	D4 D11 D11 S	D5 D5 D6 D8 S
5	.1	D6 D7 D8 D9 D11 SS †	D6 D7 D7 D11 D13
6	.1	D4 D9 SS	D7 SSSSS
7	.1	D4 D6 D6 D11 SS	D6 D6 D6 D6 D7 D8 D9 S
8	.1	D5 D6 D6 D6 SS	D3 D5 SSS
9	.1	D7 D10 SSSSSS	D5 D5 D8 SS
			SSSS to mice‡ 00000
			D7 SSS to mice‡ 0000

* Each symbol represents one egg. D indicates embryo died, and the number the day of death. S indicates survival. Underlining of symbol indicates source of material for passage.

† Indicates source of material for other egg passages not included in table. All these proved ultimately negative for virus.

‡ Each symbol represents one mouse. 0 indicates no evidence of viral activity as manifested by pulmonary lesions.

fected yolk sac suspension. The volume of inoculation was 0.2 cc, of which 0.1 cc represented the virus, and 0.1 cc the antibiotic. It had previously been shown(1) that 1.0 mg of aureomycin was enough to suppress virus multiplication completely. Therefore in a long series of experiments we explored the effects of the following dosages: 0.034, 0.067, 0.1, 0.2, 0.25, and 0.4 mg. Eggs were candled each day, and embryos dying before the fourth day discarded. Death of the embryo after the fourth day was taken as presumptive evidence of virus activity, although occasional non-specific deaths may occur at any time. It was not possible to establish the cause of death by stained preparations, as elementary bodies are usually not demonstrable in the presence of even small amounts of antibiotic, an observation first made by Wong and Cox (1) and confirmed by our own experience. Thus the presence or absence of virus could only be determined by subsequent passage in eggs without antibiotic, or by intranasal inoculation in mice of undiluted suspension.

Table II gives the result of a typical experiment in which virus was passed through embryonated eggs in the presence of aureomycin. It will be observed that in this experiment the two initial egg passages were made in the presence of 0.067 mg of aureomycin. Thereafter the dose was increased to 0.1 mg. One or more eggs in which death of the embryo took place were used as source of material for the next passage, and a control group of eggs (*i.e.,* without aureomycin) was also injected. At the end of the experiment residual virus activity was tested for by mouse as well as egg inoculation.

Altogether 12 experiments similar to that outlined in Table II have been performed, using the various dosages of aureomycin indicated above, and the results in every one have been almost identical. The virus seemed to survive in attenuated form for several passages, and then to disappear. For example, in the experiment outlined, there appeared to be little virus activity remaining after the fifth passage, and at the time of the ninth passage 2 successive attempts to demonstrate virus activity were completely negative.

Discussion and conclusions. The purpose of these experiments was to find out whether the phenomenon of acquired resistance could be demonstrated in the case of a filterable virus to the newer antibiotics. The virus of feline pneumonitis was employed, and the two antibiotics were terramycin and aureomycin. These two were used in subcurative dosages in mice following intranasal inoculation in one series of experiments, and aureomycin was studied in 12 experiments in varying dosages in embryonated eggs. In order to enhance the likelihood of the emergence of a resistant mutant, large inocula of virus were employed. Under the conditions of our experiments no evidence whatsoever of resistance could be demonstrated, but an interesting phenomenon was observed. In both the mouse series and the egg series virus seemed to persist through several passages in the presence of antibiotic in diminished amounts. Then, with no increase in the dosage of antibiotic, it died out. The significance of this finding is obscure. That it might be due to an actual increase in sensitivity of the virus to antibiotic is conceivable, although such an hypothesis is not proven by the data presented here.

Received June 6, 1951. P.S.E.B.M., 1951, v77.

Cell Proliferation and Changes in the Large Granule Fraction During Hepatic Carcinogenesis.* (18805)

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Little quantitative study has been made of cell division during carcinogenesis, and in the few cases reported, mainly of skin carcinogenesis(1), estimates of cell proliferation were based on observed changes in the mitotic index. Such estimates are subject to error due to changes in the duration of the various phases of mitosis. An extreme example of such inaccuracy is given by the early impression that the main effect of colchicine is to stimulate mitosis, while later investigations showed its main effect to be the inhibition of cell division in the metaphase(2). The liver offers advantages for the study of cell proliferation in that it is possible to calculate the total number of cells present from the number of cells per g of tissue, a procedure introduced by Brues, Drury, and Brues(3) for the estimation of replacement of liver tissue during regeneration. Price, Miller, and Miller(4,5) have demonstrated a striking depression of the protein and pentosenucleic

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* This investigation was supported in part by the Stewart Trust Fund and by a research grant from the National Cancer Institute of the Public Health Service.

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acid content of the large granule fraction in rat livers undergoing carcinogenesis by a variety of azo dyes. In almost all cases they analyzed such livers after 4 weeks of dye-feeding. Comparison of such dye-fed livers with each other and with normal livers was based on wet weight of tissue. More recently, the importance of the cell as a unit in the biochemical comparison of tissues has been emphasized by Price and Laird(6) and independently by Davidson and Leslie(7).

The present study was undertaken to follow in detail the proliferative response of the hepatic tissue and the sequence of changes undergone by the large granule fraction of the average liver cell of rats fed 3′-methyl-4-dimethylaminoazobenzene, during the early period of dye-feeding not previously reported. The significance of this period was emphasized by the finding that the depletion of the large granule fraction of the average liver cell was more extensive after 4 weeks of dye-feeding (data of Price, Miller, and Miller[†]) than it was after 4 months of dye-feeding (unpublished data of the present author). Nuclear counts on the liver homogenates were used not only to express the analytical results in terms of the average liver cell, but also to determine the total number of cells in the liver, thus permitting for the first time a quantitative description of the over-all proliferative response of a tissue undergoing malignant change.

Material and methods. The animals used were male albino rats of the Sprague-Dawley strain[‡§] weighing about 200 g at the start of dye-feeding. They were fed *ad libitum* a semisynthetic diet[‖] to which 3′-methyl-4-dimethylaminoazobenzene was added in the concentration of 0.058%. They were killed with ether at intervals of 7, 11, 14, 16, 18, 20, 22, 24, 26, 28, and 32 days after the initiation of dye-feeding. Two groups of controls were also studied: an initial control on a grain diet, and one after 30 days on the basal synthetic diet. For each analysis, the livers of 3 rats were pooled after perfusion with ice-cold 0.14 M NaCl, and squeezed through a plastic tissue mincer to remove as much of the connective tissue as possible(8). The liver mince was then homogenized by the technic previously described(8). The cellular particles were separated by centrifugation according to the method reported from this laboratory(9), except that the nuclei were washed three times. Only the large granule fraction was saved for analysis. Two samples of the whole homogenate were suspended in 3% acetic acid, and the nuclei were counted in a hemocytometer. By counting a minimum of 800 nuclei, duplicate counts were found to agree within 5%. From these counts the numbers of nuclei per g and per whole liver were calculated. To determine the nuclei per whole liver, the number per g was multiplied by the fresh weight of the liver in g, on the assumption that the cells collected after passage through the mincer (essentially the parenchymal cells) constitute the entire weight of the liver. When the weight of the mince was compared with that of the liver before mincing, it was found that the mince constitutes more than 90% of the fresh liver, and therefore little error is introduced by this assumption. The validity of the concept of the "average cell" has been discussed previously with respect to the pres-

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[†] Recalculated on a per cell basis, assuming a desoxypentosenucleic acid content per nucleus of 10.0 x 10^{-12} g, the value customarily found in this laboratory.

[‡] Obtained from the Holtzman Rat Co., Madison, Wis.

[§] The author is indebted to J. M. Price, J. W. Harman, E. C. Miller, and J. A. Miller for samples of the livers from the rats used by them in their histological study of carcinogenesis. *Cancer Research,* July, 1951.

[‖] Casein 24%; vitab 2%; salts 4%; glucose monohydrate 65%; corn oil (containing 1 part in 600 of halibut liver oil) 5%; riboflavin to bring the total content in the diet to 1.5 mg/kg.

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TABLE I. Numbers of Nuclei and Avg Cell Wt in Livers of Rats at Various Times After Feeding of 3′methyl-4-dimethylaminoazobenzene Was Initiated.

Exper. group, days	Avg liver wt (g)	Nuclei/g of liver $\times 10^6$	Avg cell wt (g $\times$ 10^{-9})	Total nuclei/liver $\times 10^6$
Initial control	12	181	5.5	2167
7	9.5	194	5.2	1833
11	9.5	188	5.3	1780
14	12	138	7.2	1653
16	9.9	229	4.4	2262
18	9.9	229	4.4	2262
20	9.9	223	4.5	2190
22	10.2	220	4.5	2230
24	9.7	229	4.4	2230
26	8.2	226	4.4	1847
28	9.7	329	3	3200
32	8.9	315	3.2	2800
Basal diet control (30 days)	11.4	171	5.9	1950

In each case 3 livers were pooled for homogenization and analysis.

ence of binucleate and polyploid cells in the rat liver(6). Protein-nitrogen by a nesslerization procedure(10), and pentosenucleic and desoxypentosenucleic acid by Schneider's method(11) were determined on the large granule fraction, and the analytical values expressed in terms of the average cell.

Results. The number of nuclei in the whole liver remained about 2000 x 10^6 throughout the first 26 days of dye-feeding, as shown in Table I, but between the 26th and 28th days there was a sudden increase of 50% in the total number of liver nuclei.

The number of nuclei per g of liver, however, rose early in dye-feeding, leveled off during the third and fourth weeks at about 226 x 10^6 per g, and then increased abruptly by 50% between the 26th and 28th days. Thus, the weight of the average cell decreased soon after the initiation of dye-feeding and leveled off during the third and fourth weeks at about 4.4 x 10^{-9} g. Between the 26th and 28th days the average cell weight again suddenly decreased to about ⅔ of its previous value, or to about ½ of the cell weight of the normal controls. This abrupt reduction in cell size corresponded precisely in time to the increase in the total number of cells noted above.

A proportionate decrease in the pentosenucleic acid and the protein-nitrogen of the large granule fraction of the average cell occurred throughout the experimental period (Table II). This decrease was at first gradual, but coincident with the increase in cell number and decrease in cell weight, a further abrupt halving occurred, with the result that the 28- and 32-day values were about the same as those recalculated from Price, Miller, and Miller, as given in Table II, and lower than those found for tumor-bearing dye-fed livers.

No appreciable desoxypentosenucleic acid was found in this fraction in any of the analyses carried out.

Discussion. During and for 4 days after the cell division which occurred between the 26th and 28th days, there must have been complete failure to achieve net synthesis of

TABLE II. Pentosenucleic Acid and Protein-nitrogen Contents of the Large Granule Fraction of Livers of Rats at Various Times After Initiation of Dye-feeding.

Exper. group	Pentose-nucleic acid g $\times$ 10^{-12} per cell	Protein-nitrogen g $\times$ 10^{-12} per cell	PNA/N ratio
Initial control	13	34	.38
7 days	6.7	26	.26
11	6.7	23	.29
14	6.7	29	.23
16	3.1	15	.21
18	5.8	21	.28
20	5.2	19	.27
22	4.9	21	.23
24	4.6	16	.29
26	6.2	20	.31
28	2.4	9.1	.26
32	2.6	11	.24
4 wk, Price* (4)	1.7	6.2	.27
4 wk, Price* (5)	1.9	8.9	.21
4 mo.†	7.2	12	.60
4 mo.†	6.8	17	.40
Hepatoma†‡	1.4	3	.47
Basal diet control (30 days)	8.5	32	.27

In each case 3 livers were pooled for homogenization and analysis.

* Avg of 2 analyses. Assuming DNA/nucleus = 10.0×10^{-12} g.

† Data from another experimental series studied by present author.

‡ Induced by 3′-methyl-4-dimethylaminoazobenzene.

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the large granule protein and pentosenucleic acid, since the decrease in the average protein-nitrogen and pentosenucleic acid to 1/2 their previous value is more than the fall to 2/3 which would result from complete failure of synthesis of these components in the 50% of the cell population undergoing division. This effect may be due to a decreased anabolism, or to an increased catabolism, or to both, since these components presumably are undergoing active synthesis and degradation. These cell divisions induced in liver cells by the feeding of a carcinogenic dye differ from those initiated in liver cells by regeneration(6). In the sudden burst of cell division which occurred 24 hours after partial hepatectomy, the increase in the protein of the nuclear and small granule fractions and the pentosenucleic acid of the small granule and supernatant fluid fractions paralleled the increase in the desoxypentosenucleic acid per nucleus. Thus, during the prophase of this cell division net synthesis of certain cell constituents occurred, and evidence was presented to show that later, during the actual division, the large granule pentosenucleic acid also doubled, with the result that the daughter cells contained the full complement of cytoplasmic pentosenucleic acid and of nuclear and small granule protein.

In large measure the results of the present study corroborate the findings of a series of cell fractionations performed at longer intervals on the livers of rats fed 2-acetylaminofluorene(12). In that study an increase of almost 100% in the total number of cells in the liver occurred between the 4th and 7th weeks of dye-feeding. However, depression of the pentosenucleic acid, protein-nitrogen, and riboflavin of the large granules to values even below those found in tumor-bearing livers was completed before the cell division occurred.

These altered liver cells show some resemblance to the average cell of a hepatoma induced by 3′-methyl-4-dimethylaminoazobenzene, with respect to the large granule pentosenucleic acid and protein content, as shown in Table II. Whereas in the large granule fraction of normal liver cells, the protein-nitrogen content is about 20 times and the pentosenucleic acid content is about 10 times that found in a variety of tumors(13), in the 28- and 32-day dye-fed livers of the present study the amounts of these components were respectively only 3 and 2 times those characteristic of the tumors.

Cell divisions occurring subsequently to that studied here were accompanied by a statistically complete reproduction of large granule substance, since the pentosenucleic acid and protein-nitrogen contents at 4 months (Table II), in livers containing tumors, were actually somewhat higher than in the livers at 4 weeks. It however seems possible that occasionally another defective cell division might occur to produce daughter cells in which the quantity of these components was once more substantially reduced. Little more than one such defective division, occurring occasionally, would be required to produce a scattering of cells in which the large granule fraction resembled that of tumors with respect to protein and nucleic acid content.

Summary. The total number of hepatic cells was determined at frequent intervals during the first month of carcinogenesis in the livers of rats fed 3′-methyl-4-dimethylaminoazobenzene; the large granule fraction was also isolated from these livers and the quantity of pentosenucleic acid and protein-nitrogen in this fraction of the average cell was determined. The total number of liver cells remained in the normal range for 26 days, but between the 26th and 28th days a sudden increase of 50% occurred. No increase in total liver mass accompanied this increase in cell number, and evidence is presented to show that no net synthesis of large granule protein or pentosenucleic acid occurred during this cell division. This cell division without increase in cell substance is compared to the cell division induced in normal liver cells by regeneration.

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Received June 6, 1951. P.S.E.B.M., 1951, v77.

A Hexokinase Inhibitor in Nerve.* (18806)

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Whole frog nerve was found(1) to possess a considerable endogenous anaerobic glycolysis, much increased by addition of glucose to the medium. We have obtained comparable results with whole frog or rat sciatics. Homogenates of such nerves, however, exhibit little glycolytic activity; nor was this increased significantly by addition of glucose or by fortification with any combination of cofactors, etc., including ATP, DPN, Mg^{++}, F^-, and pyruvate. These findings suggested that an inhibitor of glycolysis is formed or released when normal structure is disrupted. A number of inhibitors of glycolysis is present in normal tissue. Sodium has been found to inhibit purified hexokinase (18% by .06M) (2); and to decrease glycolysis also by decreasing ATP, due to increased breakdown or decreased formation from AA and phosphopyruvate(3). Mouse brain homogenates inactivate phosphoglyceraldehyde dehydrogenase; the inhibitor resembling a cathepsin obtained from beef spleen and, like it, counteracted by certain amides and amino acid esters(4). Another potent hexokinase inhibitor, sorbose-1-phosphate, is formed under the action of aldolase by condensation of 1-glyceraldehyde and dihydroxyacetone phosphate(5). Of special interest, is the hexokinase inhibitor obtained from the anterior pituitary; a protein fraction enhanced in activity by adrenocortical extract and counteracted by insulin(6). The inhibitor in nerve homogenates is a protein, which acts specifically on hexokinase and resembles the pituitary inhibitor.

Methods. Anaerobic glycolysis of rat cerebral cortex homogenates was measured by the method of LePage(7), but with the final concentration of ATP increased to 10^{-3M}. (The ATP, 90% pure, and crystalline zinc insulin were kindly supplied by the Armour Research Laboratory.) Rabbit brain hexokinase was prepared and its activity measured according to Colowick, Cori, and Slein(6), glucose disappearance proving a more reliable index of activity than either CO_2 liberation or lactic acid production. More consistent results were obtained when the reaction was carried out in the Warburg apparatus, where shaking insured optimal mixing. Sciatic and brachial nerves of adult Sprague-Dawley and Wistar rats of both sexes were used as the source of inhibitor. They were removed within 5 minutes, without cooling, after the animal was stunned by a sharp blow on the back of the head and exsanguinated. The nerves were washed free of blood, stripped of surrounding connective tissue, and homogenized in ice cold distilled water. From 100 to 150 mg of whole nerve were usually obtained per animal. The inhibitory protein was partially purified by isoelectric precipitation. 5% water homogenates were adjusted to pH 6.0 with 0.02N H_2SO_4, in an ice bath, kept standing for 3 minutes, and centrifuged at 5000 x g for 5 minutes. The supernatant was then adjusted to pH 5.4, allowed to stand 5 minutes at 0°C, and centrifuged at 10,000 x g for 5 minutes. The second sediment after one washing with ice cold distilled water and resuspension in 0.15M KCl, was ready for use. From 200 mg of fresh nerve about 3 mg of protein with an equal amount of lipid were obtained.

Results. Nerve homogenates can inhibit the anaerobic glycolysis of rat brain homogenates by an average of over 70% for 60

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* Performed under contract between the Office of Naval Research and the University of Chicago.

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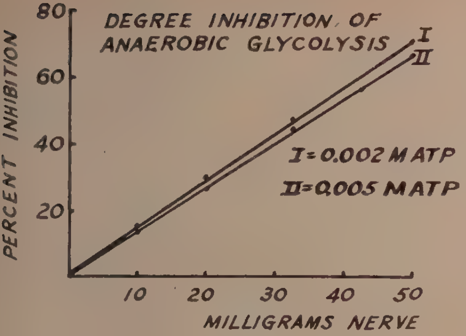

FIG. 1.

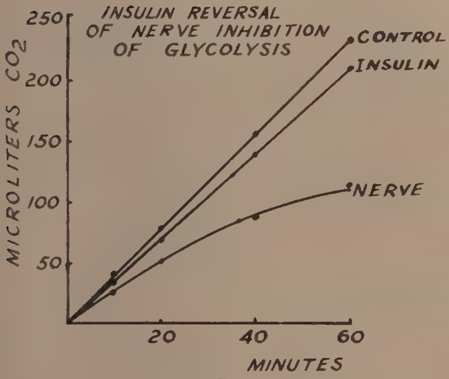

FIG. 2.

minutes, 100% at the end of this time. The amount of inhibition is linear with amount of nerve homogenate, from an average of 15% with 10 mg to 70% with 50 mg (Fig. 1). Most experiments were done with 40 mg of fresh nerve (30 mg of brain) which gave about 50% inhibition. The inhibition could be almost completely reversed by 0.5 mg of insulin (Fig. 2).

Glycolysis was at a constant rate, during the 60 minute period, in the control experiments and in those with nerve and insulin; but it dropped off rapidly in the presence of nerve homogenate only. This progressive fall of glycolytic activity cannot be due to an increased destruction of ATP by the nerve homogenate; for, at each nerve content, the inhibition of glycolysis was unchanged when the ATP was increased from .002M to .005M. (Fig. 3). Moreover, when brain and nerve homogenates, or brain hexokinase and nerve protein, were mixed an hour before adding substrate the inhibition was maximal from the start. Addition of insulin to a mixture with inhibition developed did not reinitiate glycolysis; fresh brain added to such a mixture showed at first its normal glycolytic rate. The evidence, therefore, favors a progressive inactivation of the enzyme (whether by chemical or physical change remains unknown) rather than a progressive activation of the inhibitor.

The step in the glycolytic sequence inhibited by nerve is that from glucose to hexose-6 phosphate, dependent on hexokinase. This is demonstrated by the failure to obtain inhibition of brain homogenate glycolysis with any hexose phosphate substituted for glucose as substrate (Table I; with fructose, inhibition by nerve is about half as great as with glucose), and also by the inhibition of separated hexokinase from brain. The inhibition of brain hexokinase, like that of brain homogenates, increases from 10 to 60 minutes, average inhibition for the hour being 40% with 30 mg nerve and 10 mg of enzyme (Fig. 3). Liver hexokinase is not inhibited, while that from yeast is actually accelerated by nerve homogenate.

Preliminary experiments showed the nerve inhibitor to be highly labile, so isoelectric precipitation was explored as a means of purification. Over two-thirds of the activity of the whole nerve homogenate appeared in a protein fraction isoelectric at pH 5.3 to 5.5. Two mg of this protein (not corrected for lipids present) gave a 23% inhibition of 10 mg of brain hexokinase activity; 5 mg gave almost 60%; and 0.2 mg of crystalline insulin

TABLE I. Nerve Inhibition of Brain Glycolysis with Various Substrates.

Substrate	Nerve homogenate (mg)	Inhibition (%)
Glucose	25	32
Fructose	25	19
Fructose	40	30
Glucose-1-phosphate	30	0
Glucose-6-phosphate	30	0
Fructose-6-phosphate	30	0
Fructose-1,6-phosphate	30	0

Nerve incubated with brain 20 min. before reaction of 40 min. duration. Final concentration of ATP was 0.003M. Substrate concentration was 0.01M. Each figure is avg of 5 experiments. They agreed within 5%.

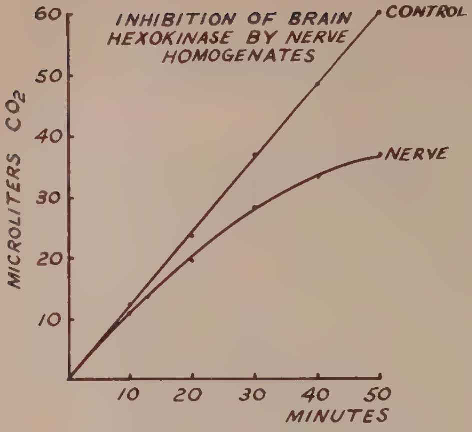

FIG. 3.

reduced this to only 20%. The values in Table II are statistically significant at the 5% level.

Heating whole nerve homogenates at 70°C (5 minutes was the minimal time tried) completely destroyed the inhibitor; even the isolated protein lost two-thirds of its activity when kept 12 hours at –5° (Table III).

Discussion. It appears conclusive from the above experiments that an inhibitor of hexokinase, a labile protein isoelectric at about pH 5.4, is present in nerve homogenates. The lability of the inhibitor, even at low temperatures, helps exclude such substances as sodium or sorbose-1-phosphate; and a spurious inhibition due to ATP-ase activity—present in the nerve protein as well as homogenate—is disposed of by addition of excess ATP. In fact, the considerable ability of crystalline insulin to reverse the inhibition tends to exclude loss of any cofactor and points to a direct action on the hexokinase enzyme.

Present evidence favors the existence of only one hexokinase in brain(8,9), so it is noteworthy that the inhibition of fructose utilization is significantly less than that of glucose. The rate of glycolysis in the presence of glucose is about 20% greater than that with fructose and is also more constant during the initial 60 minute period. These facts may relate to the difference in inhibition for the two hexoses.

Since glycolysis with fructose-6-phosphate as initial substrate is not inhibited by nerve, phosphofructokinase, which catalyzes the conversion of fructose-6-phosphate to fructose-1, 6-phosphate in the presence of ATP, is insensitive to the inhibitor.

The significance of a hexokinase inhibitor in nerve is highly conjectural. As indicated in the opening paragraph, the inhibitor seems to be formed or released when the integrity of nerve structures is destroyed. Yet some activity in whole nerve is not excluded, and this would at least fit with the 10-fold lower rate of glycolysis in nerve than in brain slices. Moreover, the inhibitor is present in the white matter of cord, at least, although less so than in nerve, while it is entirely absent from muscle and liver. The inhibitor of hexokinase obtained from nerve is strongly reminiscent of that found in the anterior pituitary by Colowick, Cori, and Slein(6). Particularly striking, is the similar isoelectric point of the nerve and pituitary factors, pH 5.4 and 5.3

TABLE II. Inhibition of Brain Hexokinase by Nerve Protein.

	No. determinations	Glucose utilized (micromoles)	Inhibition (%)
Control	10	5.6 ± .21*	
2 mg nerve protein	8	4.1 ± .20	23
5 mg ” ”	8	2.3 ± .13	59
5 mg ” ” and 0.2 mg insulin	8	4.5 ± .82	20

* Std dev. from mean. Each Warburg flask contained the following: 10 mg brain hexokinase, 1 mg glucose, .04M KF, .01M $KHCO_3$, .005M $MgCl_2$, .003M ATP. Final volume was 2.5 cc. Incubated 40 min. at 37°.

TABLE III. Lability of Nerve Inhibitor of Brain Hexokinase.

Preparation	pH of precipitation	Protein wt (mg)	Inhibition (%)
Fresh nerve protein	6	20	20
	5.4	5	59
Nerve protein 12 hr at 0°	6	20	5
	5.4	5	20
Nerve heated at 70° for 5 min.	Whole homogenate	50	0

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respectively. Both factors are also heat labile, and their effects can be counteracted by crystalline insulin. The pars distalis of the hypophysis receives unmyelinated fibers from the carotid plexus(10); it is barely possible that they constitute the source of the anterior pituitary factor. An effort is being made at present to further purify and identify the inhibitor of hexokinase found in nervous tissue.

Summary. 1. Nerve homogenates, but not those of muscle or liver, inhibit brain glycolysis, the inhibition being a linear function of the amount of nerve. 2. Two thirds of the inhibitor activity is in a protein and lipid fraction precipitated at pH 5.3 to pH 5.5. From 200 mg of fresh nerve, 3 mg of protein and a like amount of lipid are obtained. Even this semi-purified fraction loses activity slowly in the cold, almost at once on heating. 3. The inhibition is specific for brain hexokinase. The enzyme from yeast or liver is not inhibited; glycolysis of hexose phosphates by brain is not inhibited. Fructose loss is inhibited half as strongly as that of glucose. 4. Crystalline insulin can counteract the nerve inhibitor. This and its other properties are similar to those of the inhibitor from the anterior pituitary.

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Received June 7, 1951. P.S.E.B.M., 1951, v77.

Properties of a Chromoprotein Containing Extract of the Particulate Fraction of Rat Liver. (18807)

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It is generally recognized that certain reactions of terminal electron transfer to molecular oxygen are catalyzed by enzymes structurally associated with the particulate fraction of tissue suspensions. One of the difficulties in studying these reactions is the inaccessibility of tissue granules to enzyme isolation procedures. Organic solvents have been used (1,2) for the separation of catalytically active chromoproteins, associated with insoluble cell particles. It was attempted in this laboratory to use alcohols and a protective colloid simultaneously for the extraction of enzymes bound to tissue particles. The following report contains a procedure for the extraction of a catalytic system from rat liver particles, which can transfer electrons from reduced pyridine nucleotides to cytochrome C. Some physical properties of this optically homogenous system are also discussed. While this work was in progress Morton(3) reported that he could successfully employ n-butanol for the extraction of enzymes hitherto considered to be inseparable from tissue particles.

Experimental. Preparation of extract. 50 g of rat liver were homogenized in a Waring blendor for 30 seconds in ice cold solution of 0.15 *M* KCl, brought to pH 7.6 with bicarbonate. The volume of KCl solution was 100 ml. The temperature of the suspension during blending was kept at 0°C by the addition of crushed ice particles (made of distilled H_2O). The liver suspension was centrifuged between 0-4°C on the Servall angle centrifuge (type SS-1A) at 15.000 x g for 30 minutes. The supernate was decanted and the sedimented particulate fraction removed with a porcelain spoon, leaving the layer of agglutinated erythrocytes on the bottom of the centrifuge tube. The particulate fraction, or

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* Present address: The Tulane University of Louisiana, Department of Medicine and Biochemistry.

[†] This work was supported by a grant of the U. S. Public Health Service.

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sediment, was resuspended in about 120 ml KCl-bicarbonate, transferred to ice-chilled centrifuge tubes, and recentrifuged for 15′ at the same speed as previously indicated. The removal and washing of the sediment was repeated three times. After the third washing the sediment was suspended in 75 ml 0.15 *M* KCl (containing no bicarbonate) and the suspension rehomogenized. The following operations were all carried out with reagents chilled in ice bath, until their temperature was below 5°C. To the 75 ml suspension of liver particles 100 ml ice chilled isobutanol (Merck C.P.), 75 ml 0.1 *M* K-phosphate buffer (pH 7.6) and 3 ml of carboxymethylcellulose solution were added. The carboxymethylcellulose solution was prepared as follows: 0.5 g carboxymethocel-S (Viscosity X-Hi, The Dow Chemical Company, Midland, Mich.) was added with stirring to 100 ml distilled H_2O at 40°C, then heating was continued until the solution started to boil and kept at this temperature for 1 hour. The volume was made up to 100 ml with H_2O. The viscous solution was slowly cooled to room temperature and thereafter centrifuged at 4000 R.P.M. on an International centrifuge for 1 hour. This procedure removed a slightly turbid sediment, leaving a water clear supernatant, which was poured off and stored in the ice box. The mixture of liver particles, isobutanol, buffer and carboxymethylcellulose solution was vigorously shaken for a few seconds and immersed into the ice bath. A viscous light yellow paste was formed which was kept for 2 hours in the ice bath with occasional stirring, thereafter centrifuged on the Servall at 0-4°C in glass centrifuge tubes at top speed for 30 minutes. Three layers were formed, the top consisting of isobutanol, then a gummy protein cake and a reddish yellow clear bottom layer. The isobutanol was decanted, and the bottom layers were collected in an ice chilled flask. This solution, which still contained gross particles, was recentrifuged on the angle centrifuge for 60′ top speed. In some instances the centrifugation was carried out at 100,000 x g on the Spinco ultracentrifuge. In each case the resulting supernatant was a strongly fluorescent, optically clear reddish yellow solution.

Physical properties of the extract. The solution which remained clear below 10°C was inspected in a spectroscope and showed a strong band upon reduction with hydrosulfite at 560 mμ. This band diminished upon standing in air. When the isobutanol containing extract was warmed up to 14°C a dense precipitate appeared, forming a ring at the top of the solution. The precipitate disappeared rapidly upon chilling the solution in ice bath. This reversible thermoprecipitation could be repeated many times with the same solution, kept in ice bath for even a week. This dissolution of the precipitate did not occur when the temperature rose to 16-20°C. After removal of the precipitate the reduced band at 560 mμ disappeared, and the remaining strong yellow solution showed a typical flavoprotein absorption spectrum, with maxima at 450 and 469 mμ. These bands faded upon reduction with hydrosulfite. Upon removal of the isobutanol by extensive dialysis against KCl-PO_4 buffer (pH 7.6 1:1) at 4°C the thermoprecipitation no longer occurred, indicating that isobutanol precipitated, and above 16°C irreversibly denatured, a protein component of the extract. Isobutanol was most effectively removed by freeze-drying the extract. The resulting reddish yellow powder was dissolved in H_2O, and some insoluble residue centrifuged off. This solution was stable in the ice box for at least 2 weeks. The absorption spectrum of this extract was measured in the Beckman D. U. spectrophotometer (Fig. 1). Before addition of hydrosulfite a typical Soret band was found at 415 mμ, which shifted to 430 mμ after reduction, with a simultaneous appearance of a small band at 530 mμ and a marked maximum at 560 mμ. There was no spectrophotometric evidence indicating that the extract contained other compounds with heme protein like spectrum besides the one closely resembling cytochrome B.

Catalytic properties of the extract. The extract had a powerful peroxidase activity, as measured with the benzidine test, which seemed to parallel the amount of heme protein content, as measured by the degree of

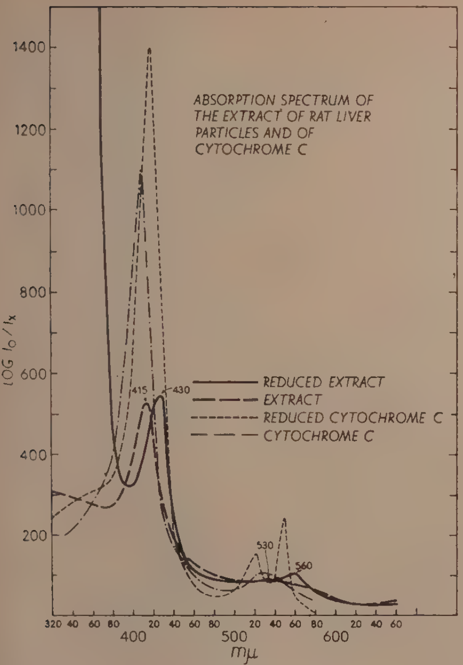

FIG. 1. Absorption spectrum of extract of rat liver particles in oxidized and reduced form compared with the spectrum of cytochrome C (3.10^{-2} micromoles). The extract is equivalent to approximately 0.5 g fresh liver. Volume of the optical system 3 ml, pH = 7.6 (0.1 M K-phosphate buffer). Optical density is plotted in terms of log $I_o/I_x \times 10^3$.

light absorption at 560 mμ upon reduction with hydrosulfite. Attempts were made to reduce the heme protein component of the extract by the yeast alcohol dehydrogenase-DPN and the Zwischenferment-TPN system. Alcohol dehydrogenase was prepared according to Racker(4), Zwischenferment as described by Warburg and Christian(5). In preliminary experiments the reduction of pyridin nucleotides by alcohol dehydrogenase or hexose 6-phosphate dehydrogenase was determined by measuring the change in optical density at 340 mμ. The reduction of 0.1 ml DPN (106 γ) by the alcohol enzyme was determined at pH 8.7, in presence of 0.1 ml glycine buffer (0.3 *M*) as heavy metal binding agent, that of TPN (120 γ 32% pure) by Zwischenferment at pH 7.4 in a final volume of 3 ml. The pH of each test system was maintained constant at 8.7 or 7.4 respectively with 0.4 ml 0.5 *M* trishydroxymethyl-aminomethane buffer(6). The dehydrogenases (0.1 ml) and substrates were in excess in each case. Carboxymethylcellulose in amounts present in the extract had no effect on the rate of reduction of the pyridine nucleotides. The enzymatic reduction of the coenzymes was complete in 30-50 seconds. The amount of extract of liver particles per cuvette was equivalent to approximately 0.4 g fresh liver. The enzymatic reduction of the extract was started by addition of either the substrate (0.1 ml 95% ethanol, or 0.1 *M* Na-glucose-6-phosphate), coenzyme, or dehydrogenases. A corresponding system, where the same component was replaced by water, served as blank. There was no change in light absorption at 430 and 560 mμ unless all the components of the reducing system (rat liver particle extract, substrate, dehydrogenase, coenzyme) were present, while a slow increase of optical density at 430 and 560 mμ occurred with both complete dehydrogenase systems. The cytochrome B-like component was under these aerobic conditions reduced by 40% only in 15 minutes. The addition of 0.1 ml cytochrome C ($3.4.10^{-2}$ micromoles) to these systems resulted in an instantaneous and complete reduction of the C component (at 550 mμ), and a subsequent increase of the reduction of the cytochrome B-like component (at 560 mμ) in 2-3 minutes. The reduction of component B in the presence of cytochrome C was about 70% with alcohol dehydrogenase-DPN, while it was complete with the Zwischenferment-TPN-reducing system. The cytochrome C reductase activity of the extract of rat liver particles was also tested by using smaller amounts of enzyme (equivalent to 50-100 mg of fresh liver). The components of the optical system in a final volume of 3 ml, were identical with the ones described previously. In each case immediate reduction of cytochrome C occurred when either chemically or enzymatically reduced coenzymes were added. Cytochrome C could

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not be replaced by methylene blue or triphenyltetrazolium chloride(7) indicating the absence of diaphorase(1) activity. In fact, the extract inhibited dye reduction. It is believed that this inhibition is partly due to the enzymatic inactivation of pyridine nucleotides by the extract. Incubation of 0.2 ml extract (equivalent with approximately 80 mg fresh liver) with 2.35 micromoles of DPN in glycylglycine buffer (0.15 *M*, pH 7.5) for 60 minutes at 37°C resulted in the appearance of 0.53 micromole of nicotinamide (determined chemically)(8), and the disappearance of 0.76 micromole of DPN (determined enzymatically)(4). No change in light absorption at 550 or 560 $m\mu$ occurred if in presence of cytochrome C and rat liver particle extract succinate, acetaldehyde, or choline-chloride were added.

Discussion. The catalytic activity of the extract of rat liver particles can be defined as an electron mediating system between reduced coenzymes and cytochrome C. The cytochrome C reductase of Haas, Horecker, and Hogness(9) isolated from yeast, links reduced TPN to cytochrome C. The DPN linked dehydrogenases of mammalian tissues also reduce cytochrome C via a reductase(10,11). The separation of these systems from mammalian tissue particles has hitherto not been reported. The circumstance that the rat liver preparation can mediate electrons both from $DPN \cdot H_2$ and $TPN \cdot H_2$ to cytochrome C would suggest that this enzyme has no specificity toward coenzymes, or that 2 separate reductases may be present in this preparation. Hogeboom and Schneider(11,12) showed that mitochondria can catalyse the reduction of cytochrome C by both $TPN \cdot H_2$ and $DPN \cdot H_2$. The participation of a component of our system, which has a cytochrome-B-like spectrum, is as yet not quite clear. The possible identity of this component with cytochrome B is based at present merely on spectroscopic evidence. Keilin and Hartree(13) suggested that attempts to "solubilize" cytochrome B may modify the heme protein. This problem can only be solved by isolation of cytochrome B, since it is difficult to see what the criterion of modification of a substance is which is defined almost exclusively by its absorption spectrum. Spectrophotometric properties of the B-like compound indicate that it can be slowly reduced enzymatically. The slow rate of aerobic reduction of component B may be due to its low E_0' (–0.040 v) (Ball)(14) and its autooxidizibility. There was some indication that the Zwischenferment-TPN system reduced the B component more readily. Addition of cytochrome C greatly accelerated the reduction of B by the coenzyme linked dehydrogenases. The sequence of the appearance of the reduced bands (first C, then B) may be explained in such a way that traces of reduced B are sufficient to maintain a steady flow of electrons to C, and B becomes visibly reduced only after C is completely in reduced form. If this explanation is valid it substantiates the view of Potter and Lockhart(15), who suggested that cytochrome B is necessary to link pyridine nucleotide dependent dehydrogenases through a flavoprotein component to cytochrome C. Slater(16) recently showed that a factor is necessary between $DPN \cdot H_2$, flavoprotein and cytochrome C. It cannot be excluded that the B component of the extract of liver particles is related to, or identical with this compound. It is of interest that variations in the procedure of extraction of liver particles altered primarily the heme protein content of the extract. Prolonged extraction (for 5-6 hours)

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or the use of less isobutanol or replacement of phosphate by bicarbonate resulted in low heme protein content of the extract. When the extraction was carried out at 30-37°C for 1 hour no heme protein could be detected spectrophotometrically. This yellow extract had diaphorase activity. The fractionation of the extract on a larger scale and characterization of the components is now being attempted.

Summary. Washed rat liver particles were subjected to treatment with isobutanol in the presence of dilute solution of carboxymethylcellulose sodium salt and phosphate buffer at ice bath temperature. The resulting extract, after being freed from isobutanol, contained a water soluble catalytic system capable of transferring electrons from DPN • H_2 or TPN • H_2 to cytochrome C. The possible identity of a heme protein-like component, with cytochrome B and other enzyme properties of the extract (peroxidase, DPN-ase) are discussed.

I am deeply indebted to Dr. Brigit Vennesland for her continuous interest and criticism in this work. The generous gift of samples of DPN, TPN, alcoholdehydrogenase, Zwischenferment and cytochrome C from Dr. Eric Conn is greatly appreciated. My thanks are due to Dr. H. G. Williams-Ashman, who determined the enzymatic breakdown at DPN.

Received June 14, 1951. P.S.E.B.M., 1951, v77.

A Method for Determining Blood Volume of the Rat Using Radioactive Phosphorus.* (18808)

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With the advent of radioactive phosphorus, methods for the determination of the blood volume using the P^{32} labeled red cells have been worked out for a variety of mammals including man. The purpose of this paper is to present a method for measuring the blood volume of the rat using P^{32} labeled red cells, and to report the results obtained.

Method. The method used is a modification of the method employed by Reid and Orr(1) for human blood volume determinations using radioactive phosphorus labeled red cells. A normal adult ether-anesthetized rat is bled to death by either aortic or cardiac puncture and the blood heparinized and centrifuged. The serum and buffy coat are pipetted off, an equal volume of physiologic saline solution containing 12 μc of P^{32}†(2) is added and gently mixed. (Either carrier or carrier-free radioactive phosphorus may be used. If carrier P^{32} is used, care must be taken to prevent the K salt concentration from becoming too high, as hemolysis will result.) The cells are incubated for 45 minutes at 37°C; then are centrifuged and washed 3 times with normal saline. The final standard suspension of cells should be approximately equal to the original volume of blood—ordinarily about 8 ml. When the determination is to be made, the rat is weighed to the nearest half-gram and lightly anesthetized with ether. The jugular bulb is exposed and a half ml of the standard labeled cell suspension injected intravenously. (For this purpose we use 27 gauge needles and half ml tuberculin syringes.) Ten minutes is allowed to elapse and one-half ml of blood is withdrawn by cardiac puncture. This unknown rat sample is laked in 2 ml of tap water in a counting chamber cup.[‡] One-half ml of the standard labeled cell suspension is diluted to 10 ml in physiologic saline. One-half ml of this diluted standard cell suspension is laked in 2 ml of tap water as the diluted standard sample. Both samples are evaporated to dryness at 95°C. The activity counts are then measured by a Geiger-Muller counter. The blood volume is calculated as follows: Example rat No. 7—wt 230.2 g. Background = 0.5 count per sec. Diluted standard sample = 8.7 counts per sec. (uncorrected). Corrected diluted standard sample = 8.2 counts per sec. per ½ ml. 20 x 8.2 = 164 counts per sec. per ½ ml = corrected standard sample. Corrected rat unknown sample = 5.7 counts per ½ ml per sec. $\frac{164}{5.7} = 28.8$ ½ ml in blood volume. $\frac{28.8}{2} = 14.4$ ml blood volume. For the purpose of this experiment, the specific gravity of the blood is assumed to be 1—thus the percentage body weight $= \frac{14.4}{230.2} =$ 6% body weight.

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* This work was done under U. S. Atomic Energy Commission Contract AT (30-1)-901 with the New England Deaconess Hospital.

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† The P^{32} used was supplied by the Oak Ridge National Laboratories, Oak Ridge, Tenn.

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This procedure may be repeated, as one does not need to sacrifice the animal. If a second determination is performed, a half ml sample of blood is obtained by cardiac puncture prior to giving the labeled red cells intravenously for the second time, and its count value subtracted from the sample obtained after the injection of the labeled cells—the calculation of the blood volume is otherwise the same.

Results. This study represents the blood volume determinations upon 34 normal adult white rats, seven of which were repeated 2 days after the first determination. The average blood volume for the whole series is 6.3% of body weight. The maximum variation in the repeated volumes is ±1.0% body weight, while the maximum variation from the average blood volume for the whole series is 1.7% body weight. The standard deviation is 0.7. 98% of the values fall within twice the standard deviation. The figures are given in Tables I and II.

TABLE I.

Rat No.	Body wt, g	Blood vol, ml	% body wt
58	175	10	5.7
99	118.2	7.1	6
2	182.8	10.6	5.8
8	137.2	8	5.85
3	232	16.3	7
1	262.2	16	6.1
2	267.1	12.5	4.6
4	271.1	13.45	4.95
5	207.8	14.05	6.75
6	210.9	12.5	5.7
54	328.3	19.7	6
35	405.6	26.2	6.5
59	330.7	22.4	6.8
55	287.7	15.8	5.45
54	236.4	16.1	6.85
66	262.9	19.3	7.1
44	258	19.3	7.4
52	279.2	18.65	6.7
57	271.2	18.85	6.95
58	295.3	21.3	7.1
7	230.2	14.4	6
8	212.2	14.9	7
9	291.2	14.4	4.95
10	245.9	15.45	6.25
11	293.2	18.7	6
12	275.8	20.5	7.4
13	210.1	14.4	6.85
49	247.4	11.4	4.6
50	185.5	13.25	7.15
54	245	16.05	6.55
56	215.4	14.3	6.65
57	150	9.75	6.5
60	147.5	10.45	7.1
63	168	12.5	7.4
		Avg	6.3
		Max	7.4
		Min	4.6
		Std dev	.7

TABLE II.

Rat No.	Body wt, g	Initial blood vol, ml	Repeat blood vol, ml	Initial % body wt	Repeat % body wt
7	230.2	14.4	13.1	6.25	5.7
8	212.2	14.9	14	7.03	6.6
9	291.1	14.4	15.7	4.95	5.4
10	245.9	15.4	14.2	6.25	5.8
11	293.2	18.7	17.5	6	5.95
12	275.8	20.5	14	7.4	6.8
13	210.1	14.4	12.3	6.85	5.8

Discussion. The results presented here agree with those previously presented in the literature by other workers using different methods. Their results are summarized in Table III. The radioactive red cell technic

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[‡] For this purpose we use screw cap bottle tops 1.7 cm diameter and 0.3 cm depth.

TABLE III.

Name	Method	Result
Cartland and Kock(2)	Dye	6 to 7.9% B.W.
Jolly and Stini(3)	Welcker	4 to 5 % B.W.
Chisolm(4)	Welcker	5.3 to 7.3% B.W.
Scott and Barcroft(5)	CO	5.4 to 7.9% B.W.
Griffith and Campbell(6)	Dye	4.1 to 5.3% B.W.
Cutting and Cutter(7)	,,	4 to 5 % B.W.
Went and Drinker(8)	,,	6.9 to 7.9% B.W.

for the measurement of the blood volume of the normal rat may thus be considered to be a reliable one.

Conclusions. 1. A method for the determination of blood volume of the rat by radioisotope dilution is described. 2. In 34 normal adult white rats, average blood volume was 6.3% of body weight, with a stand. dev. of 0.7. 3. In 7 rats, the determination was repeated with a maximum error of ±1% body weight. 4. These results are in agreement with those reported in the literature using different methods.

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Received June 15, 1951. P.S.E.B.M., 1951, v77.

Human Ovarian Grafts onto the Chorio-Allantoic Membrane of Developing Chick Embryos.* (18809)

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From the Division of Obstetrics and Gynecology, Michael Reese Hospital, Chicago.

Murphy(1) was the first to demonstrate that mammalian tissue (Jensen rat sarcoma) could live and grow actively on the chick embryo. Minoura(3) grafted ovary and testis from chick embryo and adult chickens onto the chorio-allantois of chick embryos. His conclusions indicated that these ovarian and testicular grafts remained functional and caused, in general, modification of the chick reproductive system in the direction of the type of gonadal graft. Oakley(4), studied grafts of liver from chicks, hens, and fetal rats on the chorio-allantois of chick embryos.

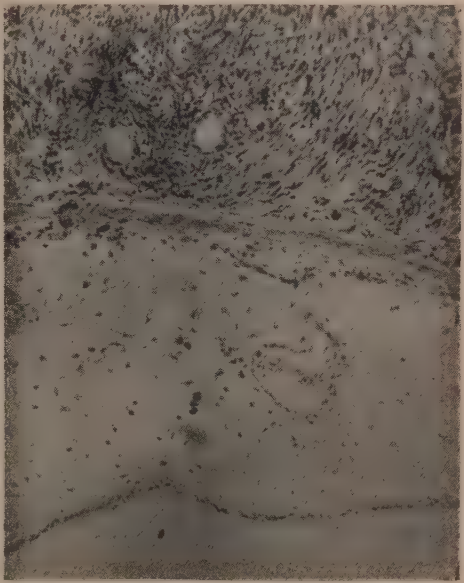

FIG. 1. Ovarian Graft, 4 Days, ×130. Follicular structures and ovarian stroma are identifiable. Contact is intimate. Vascularization absent.

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* The authors wish to express their appreciation to Dr. Marion Corrigan of the Dept. of Pathology, Michael Reese Hospital for her painstaking study of the microscopic sections and to Dr. Albert Milzer of the Dept. of Bacteriology and Virology, Michael Reese Hospital for his advice and generous use of his laboratory facilities. Mrs. Phyllis Adelman provided invaluable technical assistance.

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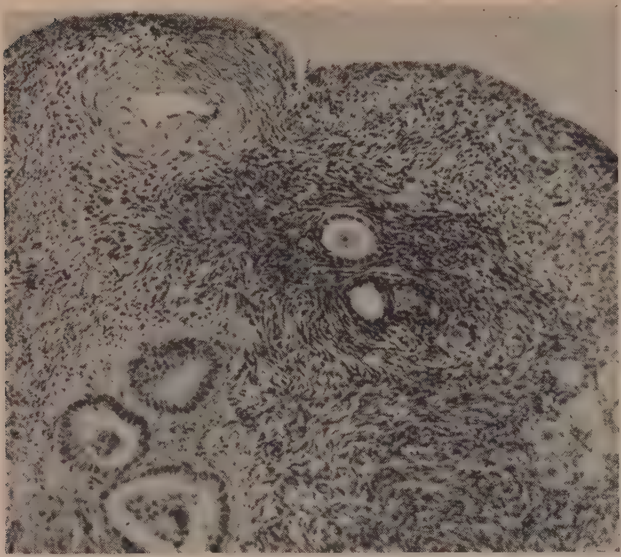

FIG. 2. Ovarian Graft, 5 Days, ×130. Primary follicles and acinar structures surrounded by ovarian stroma.

He concluded that the success of the graft and degree of growth is a function of the "individualization" (tissue differentiation? maturity?) of the transplanted material. He noted that the grafts began to fail on about the 20th day. Quoting Sandstrom, he felt that this phenomenon might be due to the progressive degeneration of the chorio-allantoic membrane as the chick approached hatching age. This is an alternate view of the one proposed by Murphy(2), who postulated that the chick embryo was incapable of a specific response to heterologous tissues until the chick was approaching hatching age.

Goodpasture, Douglas, and Anderson(5) reported the successful grafting of human skin on the chorio-allantois of the chick embryo. They describe 3 stages in the life of their grafts: 1. Stage of "plasmatic circulation". 2. Stage of vascularization. 3. Stage of regeneration and repair.

Hurst, Cook and McLennan(6) reported survival and growth of human and rabbit tissues on the chorio-allantois of the chick embryo. "Takes" were obtained with human carcinomatous tissue from the breast, gastric carcinoma metastasis to lymph glands and to omentum, inflamed salivary gland, normal lymph gland, and rabbit kidney. Goodpasture and Anderson(7) grafted human chorion and amnion onto chick chorio-allantois and succeeded in inoculating the grafts with herpes simplex, variola, and vaccinia virus. Blank, Coriell, and Scott(8) demonstrated that grafts of human skin on the chorio-allantois of developing chick embryos could be passed serially from egg to egg at weekly intervals and remain viable. Harris and Harris(9) grafted rabbit lymphatic tissue onto the chorioallantoic membrane.

Method. Seven to 10-day-old chick embryos were prepared by the "false air sac" method(10). Human ovarian tissue was ob-

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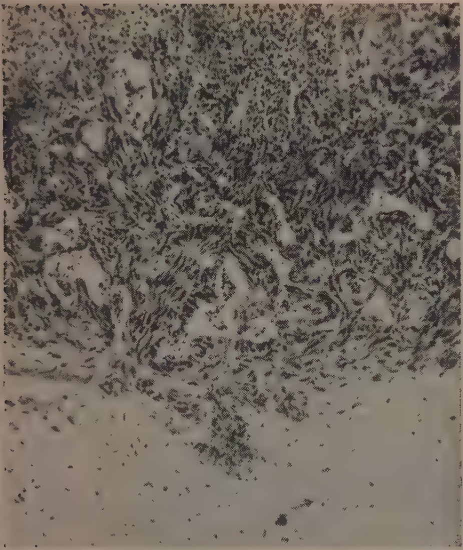

FIG. 3. Ovarian Graft, 7 Days, ×130. Vascularization of graft by capillary sprouts from chick chorio-allantoic membrane.

tained during operation. Pieces of tissue approximately 1 x 2 x 2 mm were placed in physiologic saline containing 250 units of penicillin and 250 units of streptomycin per cubic centimeter, and were introduced onto the chorio-allantoic membrane as soon as possible. The shell defects were closed either with "Scotch tape" or with sterile cover-glasses mounted on sterile vaseline. Daily observations were made with the dissecting microscope. As a rule, 7 to 10 eggs were "inoculated" at a time with the specimen of tissue to be studied. The first egg was usually sacrificed at 3 days and another egg daily thereafter. The tissues were fixed in 10% formalin, blocked in paraffin, and stained with hematoxylin and eosin. A few preparations were stained by Van Giesen and Giemsa technic.

Results. A. Ovarian Grafts. Four days after grafting, the ovarian stroma is well preserved. At the site of contact, fibroblasts are apparent. No chick erythrocytes are demonstrable within the graft. The visible follicular structures are isolated mononuclear elements, probably ova devoid of follicular epithelium, and occasional primary follicles.

After 5 days, the graft is closely apposed to the chorio-allantoic membrane. No chick erythrocytes are demonstrable within the vessels of the graft, but the capillary buds are luxuriant at the site of contact with the graft. At the graft periphery are noted ova and primary follicles in various stages of degeneration. (Fig. 1 and 2.)

Six days after grafting, the graft is en-

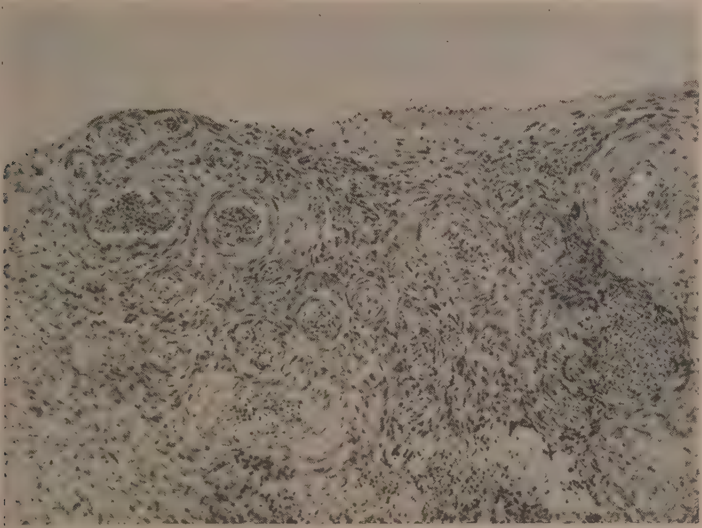

FIG. 4. Ovarian Graft, 7 Days, ×130. Intrinsic muscular walled blood vessels of graft containing chick erythrocytes.

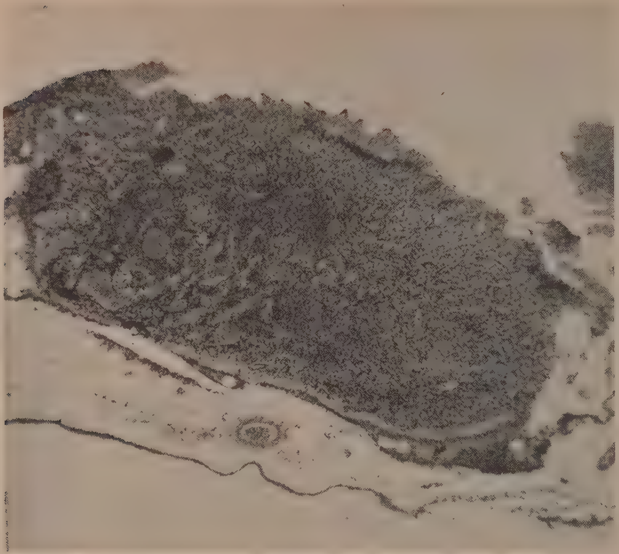

FIG. 5. Ovarian Graft, 13 Days, ×49. Extensive vascularization and early degeneration of graft.

veloped peripherally by a fine capillary network. That portion of the graft most distant from the point of contact with the chorio-allantois appears homogeneous, relatively avascular and acellular, and takes an eosinophilic stain, suggesting early hyalinization. The ovarian stroma is identifiable. Occasionally, an apparent ovum persists. Blood vessels containing chick erythrocytes extend through the graft. Many of these vessels have definite muscular walls, indicating that they are preexistent vessels of the ovarian tissue (Fig. 3 and 4.)

Eight, 10 and 13 days after grafting, the ovarian stroma remains identifiable and is vascularized. No follicular elements are seen. (Fig. 5.)

B. Endometrial Grafts. Endometrium was obtained by curettage or suction biopsy from several patients of various ages and at different phases of the menstrual cycle. Initial efforts at grafting endometrium were unsuccessful, but finally "takes" were obtained when antibiotics were eliminated from the solution into which the endometrium was placed. The specimen is proliferating endometrium obtained by curettage. After 4 days of growth, there is vascularization of the chorio-allantoic membrane. Nucleated red blood cells are seen in the vessels of the graft, and there is an intimate connection of the graft with the chorio-allantoic membrane by spindle shaped cells, loose connective tissue, and blood vessels. Well preserved cuboidal epithelial elements in glandular formation are present. The graft surface most distal to the point of contact with the chorio-allantois showed fibrin, erythrocytes and nuclear detritus (Fig. 6).

Discussion. The general pattern of development of these grafts parallels the reports from the literature. At 4 days, survival of the graft is demonstrable, even though there is no vascular connection with the chorio-allantois established. The next 4 or 5 days after grafting reveals progressive vascularization of the graft, survival (growth?) of the less "specialized" elements and degeneration of the more "specialized" elements of the ovarian tissue. As the chicks approach hatching age, there is more tissue degeneration in the grafts, and there is progressive peripheral investment of the graft by capillary networks from the chorio-allantois. At this stage, the grafts appear definitely to act as foreign bodies and to provoke a destructive action by the chorio-

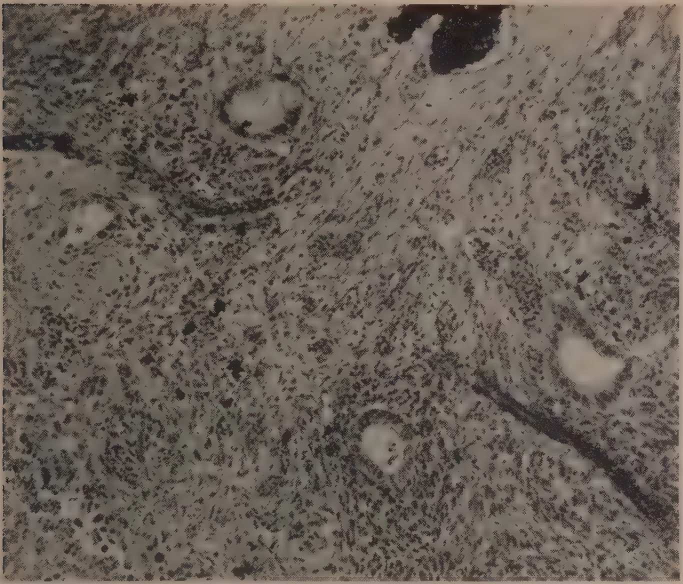

FIG. 6. Endometrial Graft, 4 Days, ×130. Carbon particles, injected into chorioallantoic membrane blood vessels, appear in vessels of graft. Meager glandular elements persist surrounded by endometrial stroma.

allantois of the chick.

Endometrial grafts follow the same general pattern of growth as ovarian grafts. However, the grafts were unsuccessful when they had been bathed in solutions containing antibiotics.

These preliminary data indicate that human ovarian and endometrial tissue will survive and grow when grafted onto the chorio-allantois of the developing chick embryo. To date, serial transfers of these grafts from egg to egg has not been attempted. The data suggests that the optimum time for serial transfer of the grafts would be from day 4 to day 8 after the initial grafting. The explanted tissue is apparently nourished by the "plasmatic circulation" prior to vascularization from the chorio-allantois. Serial transfer of the explants before the establishment of a "take" is worthy of investigation.

Conclusions. 1. Human ovarian and endometrial tissues have been grafted successfully onto the chorio-allantois of the developing chick embryo. 2. The active survival (growth?) span is short and the grafts provoke a foreign body reaction by the chorio-allantois as hatching age of the chick is approached. 3. Possible technics and investigative applications of the described methods are discussed.

Received March 2, 1951. P.S.E.B.M., 1951, v77.

Precipitation Patterns of Normal and Pathologic Blood Sera with Cationic Detergents. (18810)

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Cationic detergents of the quaternary-ammonium-salt-type form precipitating complexes with serum proteins which are soluble in an excess of either the protein or the detergent. The zone of precipitation, the so-called equivalence zone, varies with the different detergents(1-3). Similarly, the intensity of precipitation depends upon the concentration of the reacting protein. Thus characteristic peaks of precipitation are observed which broadly indicate the maximum interference, by the detergent, with the activities of certain enzymes, especially with oxidative processes(4).

Under normal physiological conditions the concentration of the proteins, as well as the other components of serum capable of reacting with detergents, remain constant within narrow limits and one would therefore expect relatively slight variations in the intensity of precipitation taking place when various samples of normal serum are treated with cationic detergents. Considerable differences in serum protein concentrations are known to exist under certain pathological conditions however, which may appreciably influence the reaction between serum and detergent. Since it seemed to us that such possible variations in the degree of precipitation could be characteristic for certain diseases, we have investigated whether physiological or pathological changes in the composition of the serum influence the rate and intensity of the serum protein precipitation when treated with a cationic detergent under standard conditions.

Experimental. To decreasing amounts of blood serum made up to 0.5 ml volumes with 0.9% NaCl solution were added 0.1 ml amounts of different concentrations of cationic detergent. The ensuing turbidities were measured at various intervals of time. *Blood serum**: Non-hemolytic blood serum from normal persons and from patients suffering from various diseases were used. *Cationic detergents*: The preliminary tests were carried out with the following compounds: Bradosol(5-7), (β-phenoxy-ethyl-dimethyl-dodecyl ammonium bromide); Zephiran(8), (alkyl-dimethyl-benzyl ammonium chloride); CTAB (9,10), (cetyl trimethyl ammonium bromide). In identical concentrations, the 3 compounds produced with serial dilutions of the same normal blood serum samples different precipitation patterns and turbidities with precipitation peaks at differing serum dilutions. With CTAB only a very slight precipitation was observed in all dilutions of serum, the turbidities obtained with Zephiran were more pronounced, while Bradosol gave a series of progressive turbidities in a characteristic and distinctive precipitation pattern (Table I).

Test procedure. In all experiments a broad range of various serum dilutions was used in order that the full range of precipitation, its pattern and the role of interfering

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* We are indebted to Dr. L. Berman, Summit, N. J., Dr. George Erdman, Overlook Hospital, Summit, N. J., and to Dr. M. Goldner, Chief of Medicine, Veterans Administration Hospital, Brooklyn, N. Y., for supplying the many samples of serum used in these experiments.

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TABLE I. Turbidity of Normal Human Blood Serum Measured .5, 2, 5, 10, 30 and 60 Min after Addition of 1% Bradosol.

Time	Ratio of serum to saline solution									
in min	10:0	9:1	8:2	7:3	6:4	5:5	4:6	3:7	2:8	1:9
.5	0*	0	0	0	1	2	3	4	2	1
2	0	1	1	1	2	3	4	4	2	1
5	2	2	3	3	4	4	5	5	3	2
10	4	4	5	5	5	6	6	5	4	2
30	5	5	5	5	5	6	6	6	5	2
60	5	5	5	5	5	6	6	6	5	2
120	5	5	5	5	5	6	6	6	5	2

* These values express various degrees of turbidity, 0 denoting no turbidity and 6 maximum turbidity. Relationship of these values to Lumetron readings is shown in Table II.

TABLE II. Turbidity Factors. Density of the standard serum-talcum suspensions of tubes 1 to 7. Measured in Lumetron Colorimeter, with 420 filter.

Density	Turbidity factor
<.3	0
.38	1
.64	2
.87	3
1.11	4
1.35	5
1.64	6
1.89	7

factors could be studied. For practical purposes, however, the following procedure was adopted: 0.5, 0.45, 0.35, 0.3, 0.25 ml amounts of serum were introduced into small test tubes (7 x 0.8 cm) and the volume made up to 0.5 ml with 0.9% sodium chloride solution. After shaking, 0.1 ml amounts of 1% Bradosol in 0.9% NaCl solution were added and the tubes again shaken. Thirty seconds, 2 and 5 minutes after the addition of Bradosol the turbidities were measured. All reactions were carried out at room temperature. *Measurement of turbidities.* Immediately upon the addition of the detergent, a turbidity is produced in certain tubes which progressively increases within the next few minutes. Since under these circumstances it was impossible to measure accurately the turbidities at 30″, 2′ and 5′ intervals by means of photo-electric apparatus, we have estimated by eye the turbidities occurring in the serum tubes, with turbidity standards, the standards consisting of various amounts of talcum suspended in serum so as to correspond to the photo-electric readings as shown in Table II.

Results. 30 seconds after addition of the detergents, undiluted normal serum (tube 1) and dilutions contained in tubes 2 to 4 remained clear, but considerable turbidity had formed in the last 2 tubes. Within a few minutes all tubes showed progressive turbidities and precipitations. The precipitation pattern of sera obtained from certain pathological cases, however, presents two consistent variations from the normal serum pattern described above. Thus the following turbidity (or precipitation) patterns can be distinguished:

1. *Normal turbidity pattern.* Observed with sera from normal patients and illustrated by the turbidity sequence shown in Table III, upper part. This pattern is constant.

2. *Abnormal turbidity patterns.* Certain sera samples obtained from patients with pathological processes such as nephrosis, jaundice, cancer, pulmonary infarction, gave either of the following turbidity patterns: a. *Abnormal turbidity pattern with heavy precipitation.* In this type even undiluted serum responds to the addition of Bradosol with the immediate development of a very heavy precipitate. As shown in Table III, middle section, very slight or no progressive precipitation is observed. b. *Abnormal turbidity pattern without any precipitation.* Certain sera, upon addition of 1% Bradosol, yielded no or very insignificant precipitation in all the dilutions tested. In some cases all tubes remained clear during the entire observation while in a few instances faint turbidities developed after 2 to 5 minutes. This pattern

TABLE III. Turbidity Patterns. Turbidities measured 30 sec, 2 min and 5 min after addition of 1% Bradosol. Factors according to Table I.

		Tube No.					
	Time	1	2	3	4	5	6
1. Normal	30 sec	0	0	0	0	1	2
	2 min	1	1	2	2	3	4
	5 ″	2	2	3	3	4	5
2a. Abnormal with heavy precipitation	30 sec	7	7	7	7	7	7
	2 min	7	7	7	7	7	7
	5 ″	7	7	7	7	7	7
2b. Abnormal without any precipitation	30 sec	0	0	0	0	0	0
	2 min	0	0	0	0	0	1
	5 ″	0	0	0	0	1	1

TABLE IV. Types of Precipitation Obtained with Various Plasma Fractions and Bradosol.

Protein, Armour & Co.	Conc., %	Reaction with 1% Bradosol
Bovine albumin	5.6	No precipitation
Fraction IV4	5.6	” ”
β globulin	1.75	Strong ” , equal to precipitation shown in Table III, 2a
γ globulin	1.75	= β globulin
Fibrinogen	.6	Extremely strong precipitation, stronger than precipitation shown in Table III, 2a

was characteristic for icteric sera and is shown in Table III, lower portion. Intermediate reactions between the normal and abnormal patterns were observed with certain sera and it was, especially in the earlier stages of this investigation, rather difficult to classify a particular reaction with either the normal or abnormal pattern.

Mechanism of reaction. Efforts were made to explain the reasons for these variations. 1. *Influence of the Bradosol concentrations on the precipitation of normal sera.* The addition of 0.1 ml amounts of 2% Bradosol solution to the serum dilutions immediately produced a heavy and rapid precipitation in all tubes including those containing undiluted serum whereas a 1% solution of Bradosol yielded a progressive precipitation and the distinctive pattern described above; lower concentrations (0.5% and 0.1%) yielded only faint turbidities. 2. *Influence of the serum concentration.* Immediately upon the addition of 1% Bradosol solution to undiluted serum from normal individuals, little or no turbidity developed, but as the serum became more diluted, a progressively increasing turbidity occurred reaching a maximum at a dilution of 1:2. In dilutions of 1:8 and higher, the degree of precipitation decreased. 3. *Reaction between Bradosol and the various normal serum constituents.* The following protein fractions were tested with 1% Bradosol. Each protein was dissolved in 0.9% NaCl solution to yield concentrations approximately similar to their concentrations in normal blood-serum and these solutions were diluted as described in the section on methods. From these experiments it may be inferred that the turbidity and precipitation of blood serum-detergent mixtures are associated mainly with the presence of globulins and fibrinogen. Determinations of pH values of the various serum-detergent mixtures indicated that the values remained constant within the dilution series of the individual sera and no significant differences were observed between normal and abnormal sera. The pH range found fell between 7.12 and 7.82. 4. *Abnormal precipitation patterns.* It seems probable that the pattern characterized by heavy precipitation is due either to a lowering of the serum protein concentration or disturbances in the normal albumin-globulin-fibrinogen ratio. Another factor responsible for immediate and heavy precipitation in certain pathological sera is the presence of increased amounts of lipids. Positive reactions were obtained in normal lipemic sera which, in the absence of lipids, gave the normal precipitation pattern.

Absence of any precipitation (abnormal pattern b) was observed in sera obtained from patients with jaundice. This form of reaction occurred also in icteric sera of nephrotic or carcinoma patients in which according to previous experience we would have expected a heavy precipitation. Ox-bile and sodium desoxycholate added to normal and strongly precipitating sera in concentrations of 2% to 0.5% completely prevented precipitation of blood serum proteins. Concentrations of desoxycholate below 0.1% also gave evidence of interfering activity. Like ox-bile(11, 12), Suramin(13, 14), Tamol N(15), soap, and sodium laurylsulfonate(3) strongly interfered with serum precipitation. All these substances are known to counteract the germicidal activity of cationic detergents. It

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is possible to titrate the concentration of precipitation-preventing substances in icteric sera by adding decreasing amounts of icteric serum to tubes containing dilutions of normal serum. One icteric serum (G.) prevented the Bradosol precipitation of the normal serum even when added to yield a final concentration of 1:4. Since a similar suppression was obtained in presence of 0.2% sodium desoxycholate, this particular icteric serum contained a precipitation-preventing factor corresponding to the strength of 0.8% of a sodium desoxycholate.

Comparison between the cationic detergent-precipitation and the coagulation of blood serum by heat. Variations in the precipitation pattern seem to be due to qualitative and quantitative changes in protein concentration. The "heat coagulation test" of serum as studied by Mayer(16), Rosenow(17), Glass(18), Huggins(19, 20), and others is based upon similar changes in the protein concentration. We have compared the results of the heat coagulation tests, performed as originally described by one of us (R.L.M.) with the precipitation test. In general, the correspondence between both tests was good, especially with normal sera and with certain samples of sera from various diseases which gave a normal precipitation pattern and a normal coagulation at 75 to 77°C. Many sera with abnormal precipitation patterns also had an elevated heat coagulation point. But we have encountered various sera in which the precipitation test gave an abnormal pattern while the serum coagulated at the normal temperature range and vice versa. We are not able at this time to explain many of these discrepancies. A similar lack of correlation has been observed also in icteric sera from nephrotic or cancer patients, with normal precipitation patterns but abnormally high coagulation points. We believe that here increased amounts of bile salts were responsible for these discrepancies. Bile salts do not interfere with the heat coagulation tests but, as shown above, strongly affect the precipitation test which is highly sensitive to an increase in bile salts content.

Discussion. The precipitation and the heat coagulation reactions are by their very nature "unspecific." From our earlier work(16) on heat coagulation we concluded that abnormal coagulation temperatures are indicative of certain disturbances in the serum protein balance but are not specific for any particular disease, in spite of the fact that sera from various disease processes, such as cancer or nephrosis, coagulate more frequently at temperatures well over 79°C than did sera from other pathological conditions. Nevertheless, heat coagulation tests have temporarily been advocated as a specific diagnostic procedure especially as a cancer test(18, 19).

The precipitation patterns are influenced by certain secondary factors as for example, the presence of increasing amounts of bile salts or lipids, which do not affect the heat coagulation of serum. Under these conditions the simultaneous exploration of both reactions in a particular case may constitute a more sensitive tool than either of these methods alone, and the confrontation of the results of both the heat coagulation and precipitation reactions may enable the clinician to eliminate and interprete certain "false normal" precipitation reactions(21).

Summary. Normal human blood serum forms a precipitate upon the addition of cationic detergents, particularly Bradosol, when tested in progressive serum dilutions up to 1:2 with a characteristic pattern of increasing turbidities. Sera from patients suffering from various diseases show entirely different patterns. This reaction may constitute a helpful clinical laboratory test.[†]

[†] After this paper was submitted for publication, two articles by R. F. Jacox *et al.* appeared in *J. Lab. and Clin. Med.*, vol. 37, 721 and 728, 1951, on a similar reaction with a cationic detergent, Octab.

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Received May 21, 1951. P.S.E.B.M., 1951, v77.

Precipitation of Blood Sera with a Cationic Detergent. A Clinical Evaluation.* (18811)

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After the technique of precipitation of blood sera with cationic detergents had been developed(1), a clinical evalution of this procedure was undertaken.

Material. A total of 294 tests were carried out in a group of 234 subjects, 43 cases being repeated one or more times for the purpose of substantiating results or detecting serial changes. Twenty-one of the cases were controls (tests run on resident doctors, nurses, etc.), while the remainder (213) were male patients hospitalized in a general medical and surgical veterans hospital. Although many cases were taken at random out of the hospital population, particular emphasis was placed on the testing of all available patients with a clinical diagnosis of malignancy, hepatic, or renal disease.

Procedure. Blood samples were drawn from the patients while in a fasting state and the sera obtained through centrifugation were refrigerated at 5°C. for an average period of 24 hours prior to testing. Whole serum and normal saline-serum dilutions of 9:1, 8:2, 7:3, 6:4, and 5:5 were in each case tested with 0.1 ml of a 1% Bradosol (Beta-phenoxyethyl-dimethyl-dodecyl ammonium bromide)† solution. The degree of turbidity of all dilutions was noted at intervals of 30″, 2′, and 5′ and compared with a set of standards of seven tubes prepared by Mayer(1). Those sera which gave a 5 to 7+ reaction in 30″, with a sustained or increased degree of turbidity at 2 minutes, were considered as positive tests. All others of lesser turbidity, as well as those considered doubtful reactions, were classed as negative. In addition to the Bradosol protein precipitation reaction, 186 of the cases were additionally tested by means of the thermal coagulation point of serum. This procedure was carried out according to the methods described by Mayer(2) and by Glass(3), 78°C. being taken as the critical temperature. Inhibition of coagulation to above this temperature was considered positive.

Results. The group of 21 normal controls, without exception, showed a negative turbidimetric pattern. Four of these had more than one test carried out during varied metabolic states, including fasting, postprandial, rest, and following exercise. Uniformly negative results were obtained in these cases and it thus seemed evident that a stable reaction exists in the sera of normal healthy individuals.

The remaining 213 cases were patients with known disease. Serial determinations were carried out in 39 of these cases, 2 to 6 tests being done at various time intervals. A stable positive or negative reaction, with two exceptions, was found to exist during the period of observation. The two cases with a varying Bradosol reaction are omitted from this series as insufficient data is available to permit conclusions regarding their significance. The data given below are thus based on the number of cases and not on the number of estimations.

Fifty-four or 25.3% of the 213 cases gave positive reactions while the other 159 cases (74.7%) revealed a negative turbidimetric pattern. Analysis of the results suggested that the distribution of the positive reactors throughout the entire series was not of a random character but rather followed a pattern.

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* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

† Bradosol was supplied by Ciba Pharm. Prod., Summit, N. J.

1. Mayer, R. L., and Eisman, P. C., Proc. Soc. Exp. Biol. and Med., 1951, v77, 452.

2. Mayer, R. L., *Klin. Wschr.*, 1922, v1, 1693.

3. Glass, George B., *Am. J. Med.*, 1950, v8, June.

TABLE I. Tabulation of the 234 Cases Tested by the Bradosol Reaction.

Malignancies	Total cases	Bradosol positive	Bradosol negative	% positive
Squamous cell carcinoma	8	2	6	25
Prostatic carcinoma	9	7	2	77.8
Lymphomas	8	3	5	37.5
Sarcoma	8	5	3	62.5
Visceral carcinoma	29	17	12	58.6
Bronchogenic carcinoma	6	1	5	16.7
Totals	68	35	33	51.5
Non-malignant disease				
Benign tumors	3	0	3	0
Nephritis	4	1	3	25
Nephrosis	5	4	1	80
Cirrhosis	13	1	12	7.7
Peptic ulcer	9	2	7	22.2
Myocardial infarction	13	3	10	23.1
Misc., infectious and degenerative disease	98	8	90	8.2
Controls	21	0	21	0
Totals	166	19	147	11.4

An arbitrary division of the total cases into malignant and non-malignant disease revealed that a significantly greater number of the 54 positive cases fell into the former group. For the purpose of presentation, as noted in Table I, the cases have been accordingly divided into the two categories of malignant non-malignant disease. The former group, 68 cases, were proven by histological study. The non-malignant cases, a total of 166, were investigated thoroughly and the clinical diagnosis adequately substantiated. Any case of doubtful diagnosis or suspected but unproven malignancy was deleted from the series.

Reference to Table I reveals that 35 or 51.5% of the malignant group had a positive reaction. Nineteen or 11.4% of the non-malignant cases, including the controls, were positive reactors. With elimination of the 21 controls from the latter group, to include only cases with pathology of a non-malignant variety, 13.4% of the total were positive. Of special interest were the results obtained in 2 varieties of non-malignant disease, cirrhosis and nephrosis. One of the 13 cases of cirrhosis was positive while 4 out of the 5 nephrotics tested were positive reactors.

The thermal coagulation point as well as Bradosol reaction was determined in 186 cases of which 53 were malignancies. Fifty-nine of the 186 cases were found to have serum which coagulated at 78°C. or over and were considered positive. This total includes 32 non-malignant and 27 malignant cases, the latter representing 51% of the malignant cases so tested. A comparison of the thermal coagulation point of serum and the Bradosol reaction showed that there existed an over-all correlation in 136 cases or 73.1%, *i.e.* both tests were either positive or negative by both methods.

Discussion. During the course of this investigation and by subsequent analysis of the final results, it became evident that any attempt to diagnose individual diseases solely by the precipitation reaction would be unsuccessful. It was equally obvious, however, that a definite trend with a consistency beyond the realm of chance was obtained in many of the conditions tested. Thus, although not of diagnostic value, the figure of 51.5% positive reactions in the malignancies is sufficiently higher than observed in the alternate group to suggest that certain variations from normal, as demonstrated by protein precipitation, do exist in malignancy. The number of cases of cancer with a positive turbidimetric pattern in this study is remarkably similar to the value obtained by Glass(3), who found by means of the thermal coagulation point of serum that 52.5% of the cancer cases tested were positive. Our efforts to correlate the positive reactions with the total proteins, albumin, and globulin, or other serum constituents in the patients tested were largely unsuccessful. It has been suggested by Glass(3), and by Huggins, *et al.*(4), that the abnormal reactions in their series were dependent upon qualitative as well as quantitative disturbances in serum albumin. While this could likewise be true for the Bradosol reaction, the work of Mayer(1) indicates that the globulin and fibrinogen components of the serum proteins may be specifically implicated

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4. Huggins, C., Miller, G. N., Jensen, E. V., *Cancer Research*, 1949, v9, March.

as the disturbed serum constituents upon which the positive Bradosol test is dependent. This possibility is further suggested by the above mentioned variation in results obtained in cirrhosis and nephrosis. In both of these diseases a gross alteration of total serum proteins exists, with the common finding of a low albumin level. Despite this fact, the serum protein precipitation, as demonstrated by the Bradosol reaction, revealed that only 1 out of 13 of the cirrhotics tested was positive while 4 out of 5 cases of nephrosis gave a positive reaction. It thus seems likely that factors other than quantitative variations in the serum albumin level are responsible for the positive Bradosol reaction.

The precipitation of blood sera with cationic detergents would thus appear to be another of the group of non-specific tests which have been described in recent years. This group includes the thermal coagulation test of Mayer(2) and the iodoacetate index of Huggins(4) which demonstrate a definite alteration from the normal pattern in malignancy as well as in an appreciable percentage of non-malignant disease. Some evidence was noted during this work, as indicated by the previously mentioned cases with a varying Bradosol reaction, to suggest the use of this test as a prognostic guide to the progression or regression of malignant disease. This possibility is at present the subject of further investigation.

Summary. 1. The sera of 234 subjects were tested with Bradosol, a total of 294 tests being carried out. 2. A significantly greater percentage of positive reactions was noted in malignancy than in non-malignant disease.

Received May 21, 1951. P.S.E.B.M., 1951, v77.

Effect of Aureomycin on Liver Fat and Liver Function. (18812)

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(Introduced by Paul György.)

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Recent observations(1-2) indicate that some patients given aureomycin or terramycin developed fatty liver. This was associated with an increase in bromsulphthalein retention in some cases. The following experiments represent attempts to study this phenomenon.

Methods. Aureomycin was administered to mongrel dogs, Sprague-Dawley rats and humans in the manner and dose indicated in Table I. Serial data from dogs and humans fed aureomycin were compared to pre-treatment data. Rats fed or injected aureomycin were compared with pair-fed or pair-injected (saline) animals. Rats and dogs were continued on commercial rations and humans on their usual diet. Histological studies including fat stains were made of liver tissue from needle biopsies(3) of dogs and necropsies from rats. Liver fat in rats was also determined chemically(4). The following liver function tests were performed on dogs and humans at weekly intervals: bromsulphthalein retention(5), serum bilirubin(6), thymol turbidity(7) and zinc sulfate turbidity(8). In

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2. Yesner, R., and Kunkel, P., *Yale J. Biol. and Med.*, 1951, v23, 299.

3. Zimmerman, H. A., Personal communication.

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5. Rosenthal, S. M., and White, E. C., *J.A.M.A.*, 1925, v84, 1112.

6. Ducci, H., and Watson, C. J., *J. Clin. and Lab. Med.*, 1945, v30, 293.

7. Maclagan, N. F., *Brit. J. Exp. Path.*, 1944, v25, 234.

8. Kunkel, H. G., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 217.

TABLE I. Effect of Aureomycin on BSP Excretion and Fat Content of Liver in Normal Rats, Dogs, and Human Subjects.

Subject	No.	Dose/ day	Route of adminis- tration	Treatment (days)	Increase in BSP retention	Increase in liver fat histologically	Liver fat % by wt chemical analysis
Dogs	4	1 g	Oral	30	0	0	—
Rats	6	0	Saline IP	28	—	Control	—
	6	2 mg	IP	28	—	0	—
	6	5	”	28	—	0	—
	6	10	”	28	—	+, —	—
	25	0[*]	Oral	30, 60, 70[†]	—	Control	13.9 ± 1.1[‡]
	25	25	”	30, 60, 70[†]	—	0	13.8 ± 1.5[‡]
Humans	3	2 g	”	30	0	—	—

[*] Control animals pair-fed.

[†] 5 animals of each group sacrificed at 30 days, 15 at 60 days, 5 at 70 days.

[‡] Mean ± std dev.

addition, cephalin cholesterol flocculation(9) and gamma globulin turbidity(10) tests were done on humans.

Results. (Table I). There were no significant changes in liver fat of dogs or rats as measured histologically and/or chemically after aureomycin administration for as long as 70 days. Two g aureomycin taken orally by three healthy young men for 30 days produced no changes in liver function tests in 2 subjects. One individual showed a change in bromsulphthalein retention from control values of 2% to 6% during therapy. This was not considered significant. There were no changes in the other tests done in this subject.

Comment. Dowling, *et al.*(11) observed increased liver fat and even death in some animals treated with large amounts of aureomycin. György, *et al.*,(12) has demonstrated that aureomycin actually prevents fatty liver and prolongs the survival of rats fed diets low in choline or methionine. On the other hand, clinical studies(1,2) indicate that some patients with hepatic disease or serious infections develop fatty liver after aureomycin administration, despite adequate diets. It is the purpose of this report to point out that these various effects were not obtained in normal dogs and rats maintained on adequate diets with levels of aureomycin dosage approximating the therapeutic range, and that no liver function test abnormalities developed in normal humans.

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10. DeLaHuerga, J., and Popper, H., *J. Clin. and Lab. Med.*, 1950, v35, 459.
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12. György, P., Stokes, J., and Goldblatt, H., *Tr. Am. Assn. Phys.*, In press.

Received June 13, 1951. P.S.E.B.M., 1951, v77.

Inhibition of Pantothenate Synthesis by Streptomycin.[*] (18813)

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During the past few years the mode of action of streptomycin has been studied by several groups. Thus, Umbreit(1), and Oginsky *et al.*(2) reported that streptomycin specifically inhibits an oxidative reaction, apparently the “oxalacetate-pyruvate” conden-

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[*] This investigation was supported in part by RG 2916 from the National Institutes of Health, Public Health Service.

1. Umbreit, W. W., *J. Biol. Chem.*, 1949, v177, 703.
2. Oginsky, E. L., Smith, P. H., and Umbreit, W. W., *J. Bact.*, 1949, v58, 747.

TABLE I. Growth Response of *S. fragilis* to β-alanine, Pantoyl Lactone Plus β-alanine, and Pantothenic Acid in the Presence and Absence of Streptomycin.

β-alanine (μg)	Pantoyl lactone† (μg)	Pantothenic acid (μg)	Concentration of streptomycin (mg)				
			0	.5	.75	1	2
0	0	0	20*	25	20	34	30
1	0	0	27	24	20	30	29
2	0	0	44	56	32	54	30
3	0	0	90	108	70	70	42
4	0	0	174	198	101	78	56
5	0	0	263	232	131	80	70
6	0	0	284	260	131	85	72
0	0	.025	31			32	34
0	0	.05	52			38	40
0	0	.075	78			63	67
0	0	.1	137			132	120
0	0	.25	200			240	219
0	0	.5	262			260	257
0	0	5	280			274	261
1	1	0	34			32	41
2	2	0	48			32	47
3	3	0	112			38	54
4	4	0	180			52	60
5	5	0	270			68	78
6	6	0	280			72	70

* Figures given are turbidity readings after 24 hr growth at 30°C. Concentrations are for 5.5 ml culture medium.

† Kindly supplied by Merck and Co., Rahway, N. J.

sation, which prevents a variety of substances from entering the terminal respiration system. Fitzgerald and Bernheim(3) presented data suggesting that this antibiotic inhibits the formation of adaptive enzymes, specifically the adaptive formation of a benzoic acid oxidase in *Mycobacterium lacticola*. More recently, Zeller *et al.*(4) showed that streptomycin inhibited the diamine oxidase reaction of *Mycobacterium smegmatis*, while Barkulis (5) reported that dihydrostreptomycin inhibits the formation of acetate and formate and metabolic CO_2 under anaerobic conditions in *Escherichia coli*.

The present paper is concerned with the synthesis of pantothenic acid by yeast, and the apparent inhibition of this synthesis by streptomycin.

Materials and methods. The organism employed was *Saccharomyces fragilis* (ATCC No. 2360). The response to *beta*-alanine and pantothenic acid was determined using the medium of Billen and Lichstein(6) modified so that it contained 1% acid-hydrolyzed vitamin-free casein (Nutritional Biochemicals Corp.). The techniques for assay were those already described(6) except that a total volume of 5.5 ml was employed. Streptomycin (calcium chloride complex-Merck or streptomycin sulfate-Squibb) was added aseptically to the medium in the desired concentrations. The assay tubes were incubated at 30°C, and growth determined after 24 hours by turbidity readings in a Klett-Summerson photoelectric colorimeter.

Results. It may be noted from the data given in Table I that streptomycin exerts a marked inhibitory effect on the growth of *S. fragilis* when this organism is grown in a synthetic medium containing *beta*-alanine as the precursor for pantothenate. In contrast, however, no effect on growth is obtained when streptomycin is incorporated into media containing preformed pantothenic acid. The concentration of streptomycin required is probably a reflection of the general resistance of yeast cells to this antibiotic and is possibly due to the relative impermeability of the cell to this compound. The data are repre-

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3. Fitzgerald, R. J., and Bernheim, F., *J. Bact.*, 1948, v55, 765.

4. Zeller, E. A., Owen, C. A., Jr., and Karlson, A. G., *J. Biol. Chem.*, 1951, v188, 623.

5. Barkulis, I. L., *J. Bact.*, 1951, v61, 375.

6. Billen, D., and Lichstein, H. C., *J. Bact.*, 1949, v58, 215.

sentative of several experiments, and suggest that this antibiotic may inhibit the synthesis of pantothenate from *beta*-alanine.

Although it is probable that sufficient pantoyl lactone was synthesized by this organism, since growth proceeded normally when *beta*-alanine was the only addition to the medium, it was considered advisable to test the effect of streptomycin on the growth of this organism in the presence of both portions of the pantothenic acid molecule, as well as *beta*-alanine and pantothenic acid separately. Pantoyl lactone itself will not support the growth of this organism. The results of such experiments (Table I) suggest that the site of action of streptomycin in this system may be the inhibition of the coupling of *beta*-alanine and pantoyl lactone to form pantothenic acid. Although this conclusion appears reasonable on the basis of the growth studies presented, other possibilities exist. No definite conclusion can therefore be made until similar studies are carried out with resting cell suspensions or cell-free systems.

Summary. The results of studies on the growth response of *S. fragilis* to pantothenic acid and its precursors, *beta*-alanine and pantoyl lactone, in the presence of streptomycin, are interpreted to suggest that this antibiotic somehow interferes with the synthesis of pantothenate.

Received April 24, 1951. P.S.E.B.M., 1951, v77.

Acquired Resistance to the Shwartzman Phenomenon.* (18814)

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Although previous investigations of the effect of repeatedly eliciting the Shwartzman reaction had been somewhat contradictory, Beeson(1) showed clearly that if the reaction is produced at 2-day intervals, successive lesions diminish in intensity and animals become completely non-reactive. This state of resistance was abolished by the injection of thorotrast. The experiments reported here were designed to further elucidate this type of resistance to the Shwartzman phenomenon.

Experimental methods. White male rabbits of mixed breed weighing 2000 to 3000 g were used. The materials employed to elicit the reaction were the P-25 polysaccharide from *Serratia marcescens* (obtained from Dr. M. J. Shear) and meningococcus culture filtrate (prepared by Dr. B. Black-Schaffer). The dosage of P-25 was 0.05 mg in 0.5 ml physiologic saline intracutaneously for preparation and 0.1 mg in 1.0 ml intravenously. In some experiments these amounts were doubled, the injected volume remaining the same. Meningococcus filtrate was diluted 1:10 with physiologic saline; 0.2 ml of this material was used for skin preparation and 0.5 ml intravenously. With this dosage about 80% of animals tested showed typical hemorrhagic lesions. The intravenous "provocative" injection was given 24 hours after skin preparation and the resulting skin lesions were noted and recorded 24 hours later. The presence of hemorrhage was the only criterion used for recording the reaction as positive. In addition to the above materials, purified typhoid antigen (obtained from Dr. H. R. Morgan), which is also capable of eliciting the Shwartzman reaction was used. When multiple skin injections were given, the first was made in the right upper quadrant of the abdomen, the second in the left upper quadrant, the third on the right just caudal to the first, etc., alternating sides of the abdomen and moving the sites posteriorly. Hair was removed with electric clippers immediately prior to injection. For passive transfer experiments, blood was ob-

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* This work was supported by the Anna H. Hanes Memorial Fund.

1. Beeson, P. B., Proc. Soc. Exp. Biol. and Med., 1947, v64, 146.

TABLE I. Animals 1-4 Given Repeated Preparatory and Provocative Injections Developed Non-Reactivity by Eighth Day While Control Animals 5-8 Had Typical Hemorrhagic Lesions When Retested on Tenth Day. After rest period of 21 days, all animals had positive reactions.

Day		1	2	3	4	5	6	7	8	9	10	rest	31	32
Animal	1	S	P+	S	P+	S	P+	S	P—	S	P—		S	P+
	2	S	P+	S	P+	S	P—	S	P—	S	P—		S	P+
	3	S	P+	S	P+	S	P—	S	P—	S	P—		S	P+
	4	S	P+	S	P+	S	P+	S	P—	S	P—		S	P+
	5	S	P+							S	P+		S	P+
	6	S	P+							S	P+		S	P+
	7	S	P+							S	P+		S	P+
	8	S	P+							S	P+		S	P+

S, .2 ml meningococcus filtrate intracut. P, .5 ml meningococcus filtrate intraven. +, skin hemorrhage. —, no hemorrhage.

tained by cardiac puncture, allowed to clot in sterile containers and after centrifugation serum was separated and stored at 4°C. The longest interval between bleeding and the use of a serum was 12 days.

Preliminary studies, an example of which is illustrated in Table I, confirmed Beeson's finding that animals subjected to repeated reactions on alternate days become non-reactive. It was found in this experiment that resistance disappeared after a rest period of 21-28 days.

Effect of repeated skin or intravenous injections only. To determine if resistance was dependent upon the development of typical skin lesions the following experiment was done. Sixteen animals, each of which had demonstrated a positive skin reaction with P-25, were divided into groups of 4 and treated as follows: Group A received repeated skin and intravenous injections of P-25 on alternate days; Group B received intravenous injections only on alternate days; Group C received skin injections only on alternate days; and Group D served as controls, remaining untreated. Group A developed lesions of diminishing severity and by the fourth set of injections (eighth day) failed to react. Groups B, C, and D were then retested. Lesions appeared only in the controls, animals of Group D. Animals of Groups A, B, and C recovered reactivity after a rest period of 21 days. Table II illustrates this study. This experiment was repeated using meningococcus filtrate with similar results, with the single exception that one of the animals given only intradermal injections showed skin hemorrhage when retested.

To evaluate the importance of the route of administration of the materials, 6 animals made resistant by repeated intradermal injections of meningococcal filtrate were retested as follows: 2 were prepared with 5 times the usual intracutaneous dose (0.2 ml of 1:2 dilution) and given the usual intravenous dose 24 hours later; 2 were prepared with the usual intracutaneous dose and given 5 times the usual intravenous dose (0.5 ml of 1:2 dilution) 24 hours later; and 2 were prepared with 5 times the usual skin dose and given 5 times the usual provocative dose 24 hours later. Positive reactions appeared in the 4 animals given the largest provocative injection; increasing the preparatory dose had no apparent effect in overcoming the resistance produced by repeated intracutaneous injections. Table III illustrates this experiment. Similar results were obtained in animals made resistant by repeated intravenous injections, namely, the non-reactivity could be overcome by employing large intravenous doses but not by increasing the intracutaneous injections. Finally, it was found that animals repeatedly given P-25 or meningococcus filtrate intravenously or intramuscularly in the dosage ordinarily employed for the skin injections developed resistance as promptly as animals given the full provocative dose.

Reactivity to heterologous materials. Four animals made resistant by repeated reactions to P-25 were tested with meningococcus filtrate; no lesions appeared. After a rest period of 21 days, all 4 rabbits exhibited large areas of skin hemorrhage when again tested with meningococcal filtrate. In a similar experi-

TABLE II. Development of Non-Reactivity in Animals Given Repeated Reactions (A1-4), Repeated Intraven. Injections (B1-4), or Repeated Intracut. Injections (C1-4) with Persistence of Reactivity in Untreated Controls (D1-4). All animals gave positive reactions when tested after 21 day rest period.

Day	1	2	3	4	5	6	7	8	9	10	rest	31	32
Animal A1	S	P+	S	P+	S	P+	S	P—	S	P—		S	P+
A2	S	P+	S	P+	S	P+	S	P—	S	P—		S	P+
A3	S	P+	S	P+	S	P+	S	P—	S	P—		S	P+
A4	S	P+	S	P+	S	P—	S	P—	S	P—		S	P+
B1	S	P+		P		P		P	S	P—		S	P+
B2	S	P+		P		P		P	S	P—		S	P+
B3	S	P+		P		P		P	S	P—		S	P+
B4	S	P+		P		P—		P	S	P—		S	P+
C1	S	P+	S		S		S		S	P—		S	P+
C2	S	P+	S		S		S		S	P—		S	P+
C3	S	P+	S		S		S		S	P—		S	P+
C4	S	P+	S		S		S		S	P—		S	P+
D1	S	P+							S	P+		S	P+
D2	S	P+							S	P+		S	P+
D3	S	P+							S	P+		S	P+
D4	S	P+							S	P+		S	P+

S, .05 mg P-25 in .5 ml physiologic saline intracut. P, .10 mg P-25 in 1.0 ml physiologic saline intraven. +, skin hemorrhage. —, no hemorrhage.

TABLE III. Development of Non-Reactivity with Repeated Elicitation of Shwartzman Reaction and Effect of Increasing Provocative Dose in Overcoming This Resistance. Increasing preparatory dose was ineffective in restoring reactivity.

Day	1	2	3	4	5	6	7	8	9	10	11	12
Animal 9	S	P+	S	P+	S	P+	S	P—	S	P—	SS	P—
10	S	P+	S	P+	S	P+	S	P—	S	P—	SS	P—
11	S	P+	S	P+	S	P—	S	P—	S	P—	S	PP+
12	S	P+	S	P+	S	P+	S	P+	S	P—	S	PP+
13	S	P+	S	P+	S	P+	S	P—	S	P—	SS	PP+
14	S	P+	S	P+	S	P+	S	P+	S	P—	SS	PP+

S, .2 ml 1:10 meningococcus filtrate intracut. P, .5 ml 1:10 meningococcus filtrate intraven. SS, .2 ml 1:2 meningococcus filtrate intracut. PP, .5 ml 1:2 meningococcus filtrate intraven. +, skin hemorrhage. —, no hemorrhage.

ment, animals rendered non-reactive to meningococcus filtrate failed to react to P-25; after a rest period of 24 days, however, typical lesions were elicited with the polysaccharide. Eight animals were given daily intravenous injections of increasing amounts of typhoid somatic antigen for 6 days (10, 20, 40, 100, 200, 400 μ). Four animals were tested on the eighth and ninth days with P-25 and 4 with meningococcus filtrate; no lesions appeared.

Failure of passive transfer by serum. Animals rendered non-reactive by repeated elicitation of the Shwartzman phenomenon at 2-day intervals were bled as soon as they failed to respond and attempts were made to transfer resistance passively to normal animals. In none of the experiments was the reaction abolished by serum transfer, nor was there any inactivation of the reacting material *in vitro* mixing with serum. These experiments may be summarized briefly. In each case, the serum was from an animal resistant to the reacting material used. 1. Three animals given 5.0 ml of serum intravenously before the preparatory injection reacted with typical skin lesions to P-25. Similarly 5.0 ml of serum given immediately before skin preparation failed to protect 3 animals against meningococcal filtrate. 2. The intravenous injection of 10.0 ml of serum 30 minutes before the intravenous injection of P-25 in 4 rabbits and meningococcus filtrate in 4 rabbits did not prevent the development of skin hemorrhage. 3. The potency of P-25 and of meningococcus filtrate as skin preparatory or intravenous provocative agents was unimpaired by mixture with serum, whether injected immediately after mixing or after incubation for 6 hours at 37°C. This experiment was performed twice, employing groups of 4 animals for each agent.

Summary and conclusions. 1. The resistance to the Shwartzman phenomenon which develops when the reaction is elicited at short intervals is transient, disappearing after a rest period of 21-28 days. 2. This resistance was found to be effective against Shwartzman active materials from heterologous bacterial species. 3. It was found that a transient state of non-reactivity could be produced by repeated inoculation of bacterial substances intracutaneously, intravenously or intramuscularly, evidence that the resistance is not dependent on the development of skin hemorrhage. 4. The resistance which develops by repetitive injections could be overcome by increasing the provocative intravenous dose but not by increasing the preparatory intracutaneous dose. 5. Attempts to transfer this resistance passively with homologous serum were unsuccessful.

Received May 17, 1951. P.S.E.B.M., 1951, v77.

Effect of Oral Thiamine Administration on Thiamine Content of the Stool.[*]

(18815)

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The present investigation was undertaken with the purpose of establishing to what extent thiamine is lost in the stool in young and old individuals when this vitamin is given by mouth in therapeutic doses. Previous studies (1-5) have shown that subjects on an ordinary diet with a thiamine content of about 1.5 mg usually excrete between 0.4 and 1.0 mg of thiamine daily in the stool. According to Alexander and Landwehr(4) about half of the fecal thiamine exists inside the bodies of the bacteria, whereas the remaining half is filterable through a Seitz filter. Of the total thiamine in the stool 10-15% is present in a non-phosphorylated form, and 85-90% as cocarboxylase. As shown by Najjar and Holt(6) individuals on a thiamine-free diet will also excrete thiamine in their stools. That the thiamine found under these circumstances is derived from bacterial synthesis is suggested by the observation that treatment with succinyl-sulfathiazole will reduce the thiamine content to zero.

When thiamine is given by mouth in excess of the amount normally present in the diet a considerable part of the extra thiamine may be recovered from the stool. This increased excretion in the feces was first observed by Schultz and coworkers(1) and has later been confirmed by Youmans and his associates(2) and by Friedemann and his group(3). It was possible for Youmans to show that the large loss in the stool of administered thiamine also occurred in patients suffering from thiamine deficiency. In the studies by Friedemann and associates up to 30 mg of thiamine could be demonstrated in the stool with a daily thiamine supplementation of 40 mg, or, in other words, three-fourths of the thiamine given by mouth was lost in the stool.

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[*] Vitamin studies in middle-aged and old individuals VIII. Funds and material provided by Hoffmann-La Roche, Inc.

1. Schultz, A. S., Light, R. F., and Frey, C. N., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 404.

2. Youmans, J. B., Patton, E. W., Kennedy, A., Monroe, S. C., and Moore, B., *Trans. Am. Assn. Phys.*, 1940, v55, 141.

3. Friedemann, T. E., Kmieciak, T. C., Keegan, P. K., and Sheft, B. B., *Gastroenterology*, 1948, v11, 100.

4. Alexander, B., and Landwehr, G., *J. Clin. Invest.*, 1946, v25, 287.

5. Denko, C. W., Grundy, W. E., Porter, J. W., Berryman, G. H., Friedemann, T. E., and Youmans, J. B., *Arch. Biochem.*, 1946, v10, 33.

6. Najjar, V. A., and Holt, L. E., *J.A.M.A.*, 1943, v123, 683.

Experimental. In the present investigation the thiamine content of the stool was determined in 12 younger individuals and in 21 old subjects, none of whom presented signs of gastrointestinal disease. The thiamine was determined by Hennessy and Cerecedo's(7) method in the modification of Friedemann and Kmieciak(8), which measures the total thiamine fluorometrically in the form of thiochrome. The prepared diet of the patients was found on analysis to contain an average of 1.5 mg thiamine daily. After the thiamine excretion in the stool on the unsupplemented diet had been determined, one tablet of 5 mg thiamine hydrochloride was given daily by mouth under supervision, and after a 4-week period of treatment the determination was repeated. Assays on the stool were finally made again 2 to 3 weeks after the discontinuation of the thiamine administration.

Results. The results of these studies are presented in Fig. 1, in which the values are expressed per 100 g of wet stool. It will be seen that the thiamine content of the stool both in the young and the old subjects increases considerably following the daily intake of 5 mg of thiamine, and that the excretion returns to the original level after the thiamine supplementation has been discontinued. If the mean daily volume of the stool is estimated to 150 g it will be noted that an amount of thiamine corresponding to ½ to ¾ of the dose given in tablet form is recovered in the stool. The lower excretion values observed in the aged patients are probably conditioned by a greater destruction of thiamine in the upper sections of the gastrointestinal tract.

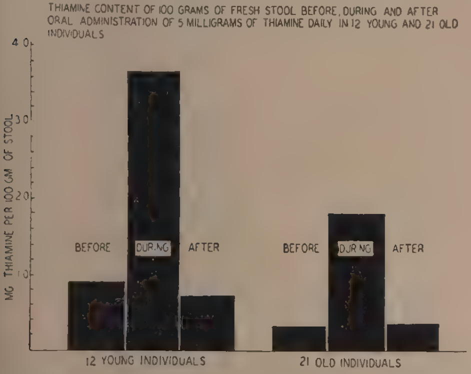

FIG. 1. The columns represent mg thiamine per 100 g of wet stool. The corresponding values expressed per 100 g of dry stool are 3.0, 9.2 and 4.3 mg respectively in the young individuals, and 1.2, 6.8 and 1.6 mg in the old subjects.

In view of the rather low absorption of thiamine from the intestine the question arises if such low absorption may be the cause of development of moderate thiamine deficiency, occasionally observed on diets considered adequate with regard to thiamine content(9). In order to elucidate this problem a comparison was made of the fecal excretion of thiamine in 24 thiamine deficient and 20 nondeficient old patients receiving an unsupplemented diet with a thiamine content of 1.5 mg daily. The thiamine blood values of the deficient group were below 2.0 μg %, of the normal group between 2.5 and 6.0 μg %. The results of the stool assays showed only a slightly higher mean fecal thiamine excretion in the patients with low blood values (340 μg per 100 g wet stool, 1640 μg per 100 g dry stool) than in the individuals having a normal blood thiamine content (285 μg per 100 g wet stool, 1285 μg per 100 g dry stool).

It is important to point out from a therapeutic aspect that in spite of the large loss of thiamine in the stool observed during treatment with thiamine by mouth, the supplementation with 5 mg of thiamine hydrochloride daily to thiamine deficient old patients resulted in an increase of the blood thiamine values to a normal level. Thus it was found(10) in observations on 20 patients, that such treatment, maintained for 4 weeks, sufficed to elevate the mean blood thiamine concentration from 1.8 to 4.0 μg % and to cause a marked improvement of various deficiency signs present. Notwithstanding these therapeutic results it would seem advisable in

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7. Hennessy, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1939, v61, 179.

8. Friedemann, T. E., and Kmieciak, T. C., *J. Lab. and Clin. Med.*, 1943, v28, 1262.

9. Kirk, J. E., and Chieffi, M., *J. Gerontol.*, 1950, v5, 236.

10. Chieffi, M., and Kirk, J. E., *J. Gerontol.*, 1950, v5, 326.

view of the large fecal thiamine loss encountered during oral thiamine treatment to administer the vitamin parenterally in those instances in which a rapid restoration of the blood thiamine level is desired.

It has not yet been established whether the excess thiamine observed in the stool after intake of commercial thiamine tablets is present in the bacteria or in the other fecal constituents; observations concerning this point would probably yield information of value. Thus if it can be shown that the excess thiamine in the stool under these conditions is non-filterable, this would suggest that the vitamin is incorporated in the bacteria and would presumably indicate that the intestinal bacteria had stolen the ingested thiamine. If on the other hand the thiamine can be recovered in the Seitz filtrate of the stool such finding would support the contention, recently proposed by Friedemann and his associates(3), that the intestine possesses a specific barrier against the absorption of thiamine.

Summary. The daily oral administration of 5 mg of thiamine hydrochloride in tablet form to 12 young and 21 old individuals was followed by an increase of the fecal thiamine excretion corresponding to one-half to three-fourths of the thiamine dose given. The fecal excretion of thiamine returned to the original level after the thiamine supplementation had been discontinued.

Received March 13, 1951. P.S.E.B.M., 1951, v77.

Chromatographic Separation of Estrone, Estradiol and Estriol.* (18816)

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Chief among the outstanding problems in clinical endocrinology is that concerned with the separation and quantitative estimation of the 3 natural estrogens. With the publication of Boldingh's(1) method for the use of rubber columns for the chromatographic separation of fatty acids it occurred to us that this technic might be adaptable to the foregoing problem. This communication consists of a report on the successful adaptation of rubber columns to the chromatographic separation of the steroids in question.

Physical characteristics of the column. The chromatographic tubes are 8 mm internal diameter and at least 20 cm in length. A short tip of approximately 3 mm diameter at the base of the tube is equipped with a piece of surgical rubber tubing ⅛ inch I.D. The flow of liquid through this portion of the tube is controlled by a small screw clamp. *Preparation of the rubber.* Pulverized mealorub rubber† is extracted with acetone in a continuous extractor for 24 hours. Thereafter, the rubber is air dried, moistened with methanol and ground in a mortar. It is then transferred to a 20-mesh sieve which is immersed in methanol. The particles which pass through the sieve are washed with the latter solvent and stored under it until used for chromatography.

Preparation of the column. Rubber columns are prepared in duplicate. These consist of a test column on which the estrogens are to be chromatogrammed and a blank control column through which only the solvents will be passed. The blank column which is submitted simultaneously to the same chromatographic pro-

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* These studies were supported by a grant from the California State Department of Mental Hygiene, Langley Porter Clinic. We are indebted also to Ciba Pharmaceutical Products for estradiol and estrone, and to Parke, Davis and Co. for estriol.

1. Boldingh, J., *Rec. trav. chim.*, 1950, v69, 247.

† Vulcanized rubber powder obtained from Andresen Corp., Chicago.

TABLE I. Spectrophotometrically Determined Recovery of Chromatogrammed Estrogens.

Rubber column	Vol. of each solvent used, ml	% error for recovery of estrogens (400 μg each)					
		Estriol		Estradiol		Estrone	
		μg	% error	μg	% error	μg	% error
1	20	370	— 8	393	—2	400	0
2		434	+ 9	417	+4	440	+10
3		353	—12	378	—6	392	— 2
4		356	—11	389	—3	403	+ 1
5	25	410	+ 3	408	+2	407	+ 2
6		440	+10	411	+3	384	— 4
7		420	+ 5	424	+6	404	+ 1
8		398	— 1	413	+3	387	— 3

cedures as those employed for the test column, provides the values on the basis of which corrections are made for solvents, rubber, etc. 1.6 g of rubber per column, prepared as described above, is shaken intermittently for 5 minutes with 35 ml of 10% aqueous methanol (10 parts methanol with 90 parts water, v/v). The resulting suspension is filtered through a fine mesh screen, and the retained particles are resuspended in 70 ml of 10% aqueous methanol saturated with benzene.‡ After adding 2 ml of benzene to this rubber suspension, and shaking the mixture intermittently for 5 minutes, it is poured slowly into the chromatographic tube. A glass bead resting on the constriction at the bottom of the tube allows the solvent to drain through the tube and at the same time prevents rubber particles from passing into the receiver. The rubber column is kept covered with solvent while it is being packed as well as during all of the succeeding elution procedures. As the rubber particles settle in the tube they are tamped gently with a stirring rod to insure their uniform packing. At this stage the rubber ordinarily fills the chromatographic tube to a height of about 12 to 13 cm. A glass wool plug is placed on the surface of the rubber column and then tamped with sufficient force to compress the column to a height of 11.5 ± 0.5 cm. The column is now drained until the surface of the solvent is just below the glass wool plug. The three estrogens, dissolved in 0.2 ml of methanol, are discharged into the glass wool from a micro pipette. The column is again allowed to drain until the sample solution passes from the glass wool into the upper part of the rubber column. An identical procedure is followed for the blank column, substituting 0.2 ml methanol for the sample solution. In addition to chromatogramming the 3 estrogens as a mixture, they were also treated in the same manner individually.

Selective elution of the column. It was found that the rubber columns, as defined, would handle 400 μg of each of the 3 crystalline estrogens, *i.e.*, a total of 1200 μg when these steroids were chromatogrammed as a mixture. These amounts are within the range of accurate determination spectrophotometrically(2). Twenty or 25 ml each of 20, 40 and 60% aqueous methanol (v/v) were passed successively through the column (Table I) at a rate of 0.4 ml per minute at 25°C. The total volumes of aqueous methanol eluate were collected in 12 or 15 successive 5 ml fractions from the bottom of the column as it developed. The number of fractions collected depended on the original total amount of the eluant. In the course of chromatogramming the mixture of the three crystalline estrogens, estriol is eluted by the first solvent passed through (20% aqueous methanol); estradiol by the second solvent (40% aqueous methanol); and estrone by the third solvent (60% aqueous methanol) when 20 ml of each solvent is used. The observations made as to the order of elution of the estrogens when they were chromatogrammed individually were confirmed by subjecting these steroids to the color tests developed by Bachman(3), Szego and Samuels(4), and the Zimmermann as modified by Friedgood, *et al.*(5). When 25 ml

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‡ Redistilled, thiophene-free.

2. Friedgood, H. B., Garst, J. B., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1948, v174, 523.

3. Bachman, C., *J. Biol. Chem.*, 1939, v131, 463.

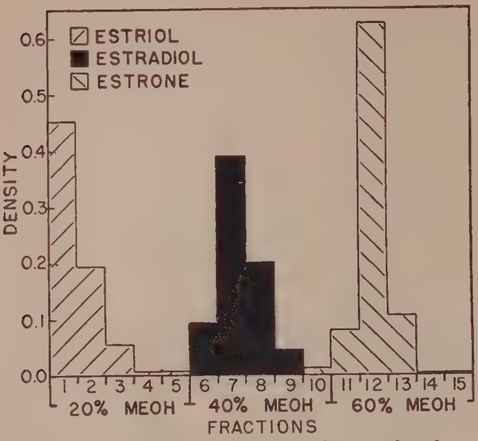

FIG. 1. Corrected densities at 280 mμ for fractions obtained from chromatographic column No. 5 (Table I). Each fraction represents 5 ml of eluant.

$$\text{Density} = \log \frac{I_o}{I_x}$$

of each eluant is passed through the column, fractions 1 through 5 contain estriol; fractions 6 through 9 contain estradiol, and fractions 10 through 15 contain estrone. In order to determine the nature of the estrogen in fraction 10 (Fig. 1), estrone and estradiol were chromatogrammed separately. It was found that fraction 10 contains only estrone. This fraction was therefore grouped with fractions 11 through 15, containing the major part of this estrogen. In view of the fact that crystalline estrogens are more soluble in 80% aqueous methanol than in 20, 40 or 60% concentrations, the columns were treated subsequently with 80% aqueous methanol in an attempt to determine whether or not a measurable quantity of estrogen remained uneluted. Within a few minutes after the addition of the 80% aqueous methanol, the rubber contracted visibly, coincidentally with the release of the bound benzene in the column. Spectrophotometric analysis of the eluate that was collected before and after contraction of the column failed to disclose a measurable quantity of any of the 3 estrogens.

Analysis of eluted fractions. Each of the foregoing 12 or 15 fractions was taken to dryness and dissolved in 4 ml 95% ethanol. The ultraviolet absorption of each fraction was determined at 280 mμ in a Beckman spectrophotometer. These values are shown in Fig. 1 and 2. Table I shows the per cent error for the recovery as determined by spectrophotometric analysis of the 3 estrogens eluted from rubber columns as described above. It is clear from these data that known amounts of a mixture of the three natural estrogens can be separated sharply from each other and estimated quantitatively within an experimental error of approximately ± 10%. These data have demonstrated, moreover, that one can determine the identity of an unknown estrogen by the concentration of the aqueous methanol which will remove it from a rubber column.

It should be noted that the use of the rubber column described above is not limited to the amount of estrogenic material employed in the present investigation. The accuracy with which various amounts of estrogens can be recovered from these columns and determined quantitatively will be dependent largely on the accuracy and sensitivity of the assay method employed. This chromatographic procedure can be applied to the separation of both larger and smaller quantities of estrogen by appropriately varying the amounts of rubber and solvent employed.

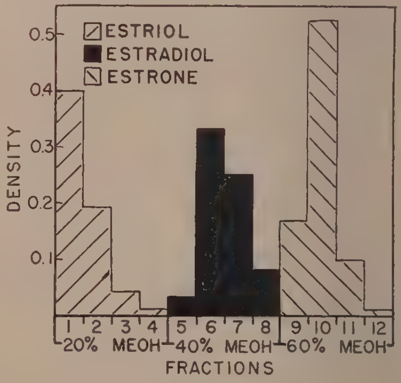

FIG. 2. Corrected densities at 280 mμ for fractions obtained from chromatographic column No. 1 (Table I). Each fraction represents 5 ml of eluant.

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5. Friedgood, H. B., and Whidden, H. L., *Endocrinology*, 1940, v27, 249.

Summary. A column of pulverized Mealorub rubber has been adapted successfully to the quantitatively accurate chromatographic separation of a mixture of crystalline estrone, estradiol and estriol. When 20 ml each of 20, 40 and 60% aqueous methanol (v/v) are passed successively through the column, estriol is eluted by the 20%; estradiol by the 40%, and estrone by the 60% concentration of methanol.

Received May 3, 1951. P.S.E.B.M., 1951, v77.

A Non-specific Brucella Agglutinating Substance in Bovine Serum.*† (18817)

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In evaluating the results of the serum agglutination test for brucellosis in cattle, animals with titers ranging from incomplete agglutination at the 1:50 dilution to incomplete at 1:100 are designated as suspects, and herds containing one or more such animals, with all other animals negative to the test, are considered as suspicious or suspect herds. Animals with higher titers are considered infected and are designated as reactors. In a brucellosis control plan in Minnesota which is based on county areas and requires periodic testing of all the cattle in the controlled counties, a ratio of approximately two suspect herds to three reactor herds is usually disclosed. In counties where the percent of infection is very low, the number of suspect herds may exceed appreciably the number of reactor herds. Moreover, the majority of suspicious herds are so classified because of the presence of a single suspect animal. These observations coupled with the fact that approximately 85% of the animals displaying suspicious titers in suspect herds are found to be negative to subsequent retests have suggested that non-specific agglutinins may be responsible for a large portion of these reactions.

In the course of trying to demonstrate non-specific agglutinins for Brucella a filter paper chromatographic technic was devised that made possible the detection of an agglutinating substance capable of reacting with Brucella test antigen but differing markedly from specific or high-titered agglutinins in its affinity for adsorption on cellulose. The test was based on two recently published observations; first, Franklin and Quastel(1) showed that certain proteins together in solution could be separated by filter paper chromatography when the proper developing solution and a means of localization of the fractions were employed; and second, Castaneda(2) observed that Brucella agglutinating serums were capable of fixing a stained Brucella antigen to the surface of filter paper.

Materials and methods. Filter paper. The paper used in this test was S & S 589, White Ribbon. It was cut in strips 3½ inches long and ¾ of an inch in width. *Developing solution.* The solution found to give the best results was potassium acid phthalate (0.05 M) adjusted to pH 6.2 with sodium hydroxide. *Antigen.* A hematoxylin-stained Brucella antigen prepared according to the method of Roepke(3) for use in the milk and cream ring test was used exclusively in the test. The antigen was found to give better results if the

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[*] Paper No. 2296—Scientific Journal Series, Minn. Agric. Exp. Station, University of Minnesota.

[†] These studies were made possible by a grant from the Bureau of Animal Industry, U. S. Department of Agriculture.

[‡] Part of thesis submitted by Mr. Hess to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology and Immunology.

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2. Castaneda, M. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 46.

3. Roepke, M. H., *Brucellosis*, A.A.A.S., Washington, D. C., pp. 127, 1950.

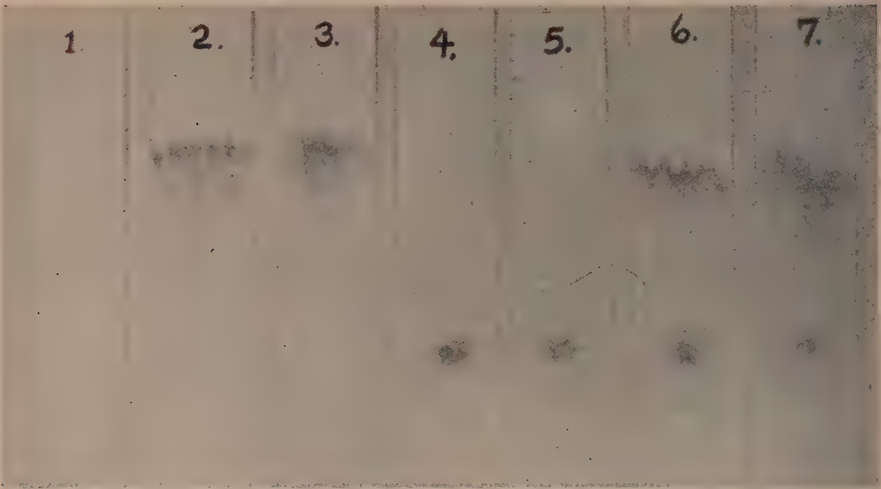

FIG. 1. Chromatographic tests for specific and non-specific Brucella agglutinating materials in bovine serum. No. 1 negative, No. 2 and 3 specific reactions, No. 4 and 5 non-specific reactions and No. 6 and 7 mixed reactions.

glycerin was removed by washing with saline in the centrifuge and resuspending to the original concentration in 0.5% phenolized physiological saline.

The test was performed in the following manner: 1. The paper strip was placed on a smooth, nonabsorbent surface. Using a 0.2 ml serological pipette held in a vertical position with its tip pressed firmly against the paper, 0.02 ml of serum was placed about an inch from one end of the strip and allowed to soak slowly into the paper. This resulted in the formation of a spot about 18 mm in diameter. The strip was hung with the tip, just below the serum spot immersed in the developing solution. It was then allowed to develop by capillary ascent. 2. After 10 to 15 minutes, the strip was removed from the solution, and by means of a small camel's hair brush the antigen was applied to the entire path of migration of the serum. It was necessary to prevent the antigen from contacting the dry area of the paper, for from there it could not readily be washed. 3. The strip was placed immediately on a towel or other absorbent surface and washed. The washing was accomplished with 1% saline applied with a second brush in a dabbing or blotting motion. Areas of the strip where antigen and antibody had combined could withstand vigorous and prolonged washing without removing all of the stained antigen.

Fig. 1 illustrates the types of reactions that were encountered. Strip 1 shows the reaction obtained with negative serums. All of the antigen was readily washed from the paper. On strips 2 and 3 the agglutinins advanced up the paper with the solvent as was evidenced by the fixing of the antigen near the top of the strips. This type of reaction was designated as specific, for it was the type given by high-titered bovine serum as well as the majority of low-titered serums from infected herds and also by serums of animals treated with Strain 19 Brucella vaccine. Serums from humans suffering from brucellosis as diagnosed clinically and confirmed by agglutination and blood culture also give this type of reaction. On strips 4 and 5 the agglutinating material remained at the spot where the serum was applied. The reaction was designated as non-specific because the agglutinating substance involved was found to adsorb on a variety of cellulose materials, and in this respect differed from the specific or gamma globulin antibody. Quite a large number of serums were found to display both kinds of agglutinins. Strips 5 and 6 serve as examples.

TABLE I. Summary of Differential Tests on Bovine Serums.

Source of serum	No. of animals tested	Filter paper reaction No. specific	 No. nonspec. only	% non-spec. only
Suspects from reactor herds	197	188	9	4.6
Suspects from suspect herds	220	79	141	64
Negatives from negative herds	100	0	12	12

A similar reaction was obtained by mixing serums of the specific and non-specific types.

Experimental. The following bovine serums were tested by the filter paper chromatographic method: 197 suspect serums from animals in herds which also contained reactors, 220 suspect serums from animals in otherwise negative herds, and 100 samples that were adjudged negative on the basis of the routine plate agglutination test in which 1:25 was the lowest dilution tested.

Results. Because a significant percentage of serums showing the specific reaction on filter paper also contained some non-specific agglutinating material, the non-specific was considered to be of importance only when it was the sole cause of the fixation of antigen on the paper. The results recorded in Table I adhere to that criterion.

It may be observed that 64% of the suspect serums from animals in otherwise negative herds gave the non-specific reaction only, while the same was true for less than 5% of the suspect serums from herds containing reactors.

Because 12 of the so-called negative serums gave non-specific reactions on paper, a portion of that group was subjected to a tube agglutination test beginning at a dilution of 1:4. The results indicated the sensitivity of the filter paper test to be equivalent to approximately the 1:16 dilution of the tube test.

The reliability of the test is being checked by following the serum agglutination retests on suspect animals from otherwise negative herds. This phase of the study is still in progress. However, the results obtained thus far are worth reporting. Of 53 suspects that gave non-specific reactions on the original herd test, 50 were declared negative to the serum agglutination retest. Two retained a suspicious titer, and 1 was reported as increasing in titer to the reactor level. This increase in titer was from incomplete agglutination at 1:100 to complete at 1:100. In contrast to the above results, out of 34 suspect animals in otherwise negative herds that originally gave specific reactions on filter paper, 16 have become negative, 11 have remained suspicious and 7 have become reactors.

Discussion. It is believed that the non-specific agglutinating substance is present in varying amounts in a large portion of animals of a variety of species. Its presence has been detected, as reported here, in a large number of bovine serums. Work still in progress indicates that more than 90% of normal swine contain the substance in detectable amounts in their serums. A similar substance also appears to be present in human serums. These latter findings are being expanded.

Attempts are being made to obtain the non-specific agglutinating material in an amount and state of purity that will enable further characterization of the substance. Adsorption on cellulose or Brucella organisms followed by elution with distilled water has yielded preparations showing a marked increase in concentration as indicated by agglutination titer. In a second line of approach, the methods for the fractionation of plasma presented by Cohn *et al.*(4) have been used. The non-specific substance is not extracted with the gamma globulin fraction as is the case with specific Brucella agglutinins. Instead, the substance with its agglutinating activity still intact is found in the fraction which Cohn has designated as containing isoagglutinins, prothrombin, and cold insoluble globulins.

The fact that readily demonstrable amounts of the non-specific agglutinating substance are present in many serums of apparently normal animals indicates that it may be a normal

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4. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahut, F. W., Leever, W. F., Lui, C. H., Mittelman, D., Mouton, R. F., Schmed, K., and Uroma. E., *J. Am. Chem. Soc.*, 1950, v72, 465.

serum constituent. In a low percentage of animals, sufficient amounts are present to cause interference with the application of the standard agglutination test at the 1:50 and 1:100 dilutions.

Summary. A filter paper chromatographic technic was used to demonstrate the presence in animal serums of a non-specific agglutinative material for Brucella. As judged by the physio-chemical properties of the material it does not seem to be related to agglutinins which arise from a specific antigenic stimulus of the classical type.

The authors wish to express their indebtedness to Dr. Fred C. Driver, Veterinarian in Charge, U. S. Bureau of Animal Industry and members of his staff, Drs. L. B. Clausen, E. H. Braunworth, F. R. Cameron, Louis Olson, Alfred Peterson and D. N. Werring for their very valuable assistance in supplying low-titered bovine serums, and to Drs. Henry Bauer and Anne C. Kimball of the Minnesota State Board of Health for providing the specimens of human serum used in this study.

Received May 28, 1951. P.S.E.B.M., 1951, v77.

Augmentative Effects of Steroids and Chorionic Hormone on Rat Ovary.
(18818)

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It long has been known that the preliminary administration of estrogenic substances results in a marked augmentation of ovarian weight induced in rats by a gonadotrophic hormone. This was first accomplished in hypophysectomized rats(1-3), and in immature females not only with estrogens(4) but with testosterone propionate(5). During the war years Dutch students(6) also approached this problem and from their results drew an elaborate theory regarding gonadal function. The purpose of the present report is to direct attention to these studies and to add further findings with various steroid substances.

TABLE I. Chorionic Hormone 750 I. U. Given on Day 2 to Each Group.

Day 1	No. Animals	Avg body wt, g	Uterus, g	Ovaries, g
Sesame oil .1 cc	4	42	.140	.023
Cottonseed oil .1 cc	5	40	.113	.024
Sesame oil .5 cc	4	46	.105	.037
Pregnenolone 5 mg	4	44	.131	.032
Cholesterol 25 ”	4	43	.150	.042
Stilbestrol .5 ”	5	39	.075	.059
” 1 ”	4	44	.083	.071
Estrone 5000 I. U.	4	46	.119	.039
” 10000 I. U.	4	49	.081	.059
Estradiol benzoate .8 mg	4	44	.095	.072
” dipropionate 2 mg	4	44	.114	.060
Progesterone 5 mg	5	38	.145	.018

The rats employed in this study were from the Stanford colony and weighed from 35 to 40 g at 21 to 24 days of age. The usual procedure was to administer a single subcutaneous injection of the steroid or control material on the first day of the experiment while on the second day they were given 3 intraperitoneal injections of 250 I.U. of chorionic hormone (A.P.L.—Ayerst). The 2 sites of injection were used in order to avoid any change in the rate of absorption of the active material. The rats were sacrificed with natural gas on day 6 when body, uterine and ovarian weights were obtained, and the ovaries fixed in Bouin's solution for section and microscopic examination.

The injection of sesame or cottonseed oil 0.1 cc on the first day of the experiment gave ova-

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TABLE II. Chorionic Hormone 750 I. U. Given Each Group on Day 1.

Day 2	No. Animals	Avg body wt, g	Uterus, g	Ovaries, g
Stilbestrol .5 mg	4	45	.099	.038
,, 1 ,,	4	42	.093	.031
Progesterone 5 mg	4	50	.141	.060

ries of .023 and .024 g while they weighed .037 g with sesame oil 0.5 cc, .032 with pregnenolone 5.0 mg and .042 g with cholesterol 25.0 mg (Table I). On the other hand, a marked increase (up to .072 g) in ovarian weight was obtained with high dosages of estrogenic substances, for instance, stilbestrol, estrone (Theelin-Parke Davis), estradiol benzoate (Ovocylin benzoate-Ciba) and estradiol dipropionate (Ovocylin dipropionate-Ciba) (Table I). With progesterone (Proluton-Schering) no enhancement of ovarian weight occurred. In all instances the histologic picture of the ovaries was the same as that found when the chorionic hormone was given alone and showed developing graafian follicles, corpora lutea and hypertrophy of the interstitial cells.

If the order in which the hormones were administered was reversed, namely the chorionic hormone on the first day and stilbestrol on the second, there was no increase in the ovarian weight (Table II). However, a considerable increase was obtained when progesterone was given on the second day (Table II).

Summary. The preliminary administration of high dosages of stilbestrol, estrone, estradiol benzoate and estradiol dipropionate resulted in a marked increase in the ovarian weights of immature rats induced by a chorionic gonadotrophin. The control series were given sesame oil, cottonseed oil, pregnenolone, cholesterol or progesterone. The increase also did not occur if stilbestrol was administered after, instead of before, the chorionic hormone. Although progesterone did not produce increased ovarian weight when given before, a marked enhancement did result when it was administered after, the chorionic hormone.

Received May 29, 1951. P.S.E.B.M., 1951, v77.

Relative Role of Milk Agent and Tissue Sensitivity in Estrogen-Induced Mammary Growth.* (18819)

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The response of the mammary gland to estrogenic stimulation varies in different strains of mice(1,2). Whether or not the presence of the milk agent is correlated with these differences is still a moot question(3-5). The following experiments constitute another attempt to contribute to the solution of this problem.

Material and methods. Ninety-eight sexually immature mice of closely inbred strains were ovariectomized at the age of 4 to 5 weeks and kept on a standard diet of Purina Laboratory Chow and water *ad libitum*. Thirty-one mice of strain Z (C_3H)—15 with the agent (Z) and 16 without it (Zb)—28 mice of strain

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* The investigation was supported by research grants from the National Cancer Institute, U. S. Public Health Service, American Cancer Society upon recommendation of Committee on Growth of National Research Council, and Graduate School of University of Minnesota.

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TABLE I. Tabulation of Microscopic Findings in Mammary Glands According to Grades.

Strain	Injected for wk	# of breasts studied	# of ducts in one low power field 48 mm obj., 10× ocul.	% of mammary glands showing stimulation of growth grade: 0	I	II	III
Z	1	8	17-35	0	50	50	0
	2	8	20-32	0	0	100	0
	4	8	25-35	0	0	0	100
	6	8	14-42	0	12.5	12.5	75
	8	12	21-41	0	0	0	100
	13	8	18-33	0	0	25	75
Zb	1	8	8-20	12.5	87.5	0	0
	2	8	4-25	25	75	0	0
	4	8	8-28	12.5	50	37.5	0
	6	6	18-38	0	33.3	50	16.7
	8	15	21-39	6.7	13.3	33.3	46.7
	13	8	21-37	0	12.5	37.5	50
D2	1	8	8-21	37.5	62.5	0	0
	2	8	8-26	12.5	50	37.5	0
	4	6	8-31	16.7	33.3	50	0
	6	8	10-30	37.5	37.5	25	0
	8	8	12-28	0	25	75	0
	13	8	14-31	0	12.5	50	37.5
D2b	1	8	4-18	62.5	37.5	0	0
	2	8	8-18	75	25	0	0
	4	8	8-30	12.5	62.5	25	0
	6	8	6-22	12.5	75	12.5	0
	8	8	17-39	0	25	75	0
	13	8	14-25	0	50	50	0
A	1	8	14-32	25	75	0	0
	2	8	6-31	12.5	37.5	50	0
	4	8	14-42	0	25	75	0
	6	8	15-41	0	25	25	50
	8	8	14-43	12.5	25	37.5	25
	13	8	12-38	0	0	62.5	37.5
Ax	1	8	9-21	50	50	0	0
	2	8	12-26	12.5	87.5	0	0
	4	8	12-35	0	62.5	37.5	0
	6	8	13-39	0	62.5	37.5	0
	8	8	12-35	25	25	37.5	12.5
	13	8	15-42	0	25	25	50
Axa	1	8	4-23	75	25	0	0
	2	8	12-25	25	37.5	37.5	0
	4	8	14-33	0	50	50	0
	6	7	25-41	14.3	28.6	57.1	0
	8	8	10-43	0	37.5	37.5	25
	13	8	18-33	0	0	62.5	37.5

D2—14 with the agent (D2) and 14 without it (D2b)—and 39 mice of strain A—14 with the agent (A), 11 without (Ax), and 14 (Axa), whose ancestors had been raised without the agent for thirty-two generations and subsequently been refostered to reacquire the agent —were used. In each group, 2 animals served as ovariectomized controls; the remaining ones received subcutaneous injections of 0.03 mg of alpha estradiol† benzoate in sesame oil once a week for 1, 2, 4, 8, or 13 weeks. One week after the last injection, the mice were sacrificed; from each animal, 4 mammary glands, uterus and vagina were removed, fixed in Bouin's solution, embedded in paraffin, and semiserial sections stained with hematoxylin and eosin were prepared for microscopic examination.

Microscopic examination. In each breast of each animal (unless stated otherwise in Table I), the number of ducts in a low power field (48 mm obj., 10x ocul.) were counted, and

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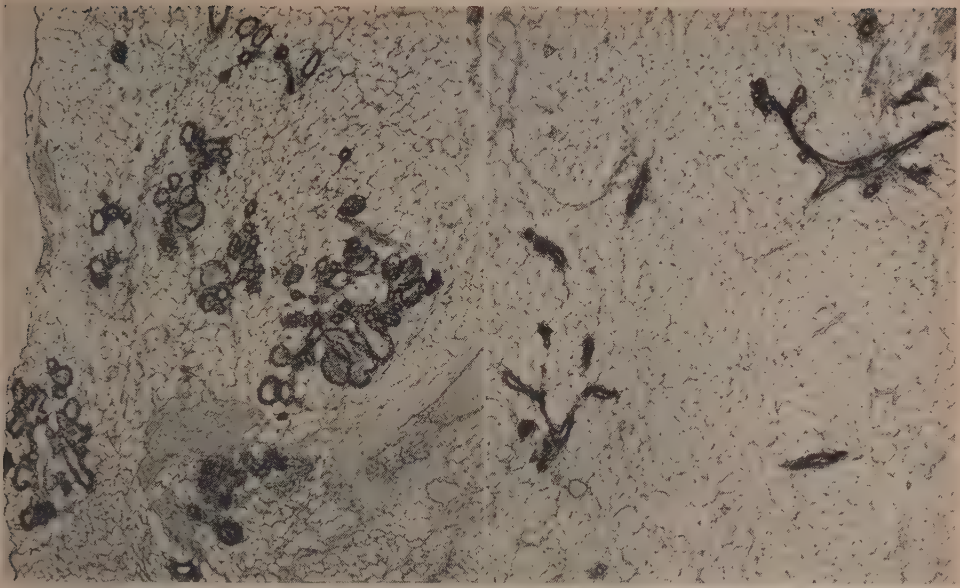
† We are indebted to Schering Corp. for generous supply of Progynon-B.



Sections through the mammary gland of mice of strain Z (Fig. 1) and Zb (Fig. 2), 8 weeks old, ovariectomized at the age of 4 weeks, and having received 4 weekly injections of 0.03 mg of alpha estradiol benzoate. The figures demonstrate the maximum degrees of stimulation observed at this experimental stage. Magnification 55×.

FIG. 1 (left). Stimulation of grade III.

FIG. 2 (right). Stimulation of grade II.

epithelial growth was studied; the findings were graded, as described previously in detail (2b). Briefly, this is the gradation: *Grade O:* Resting ductal epithelium and absence of alveoli. *Stimulation grade I:* Proliferation of ductal epithelium with papillary infolding, elongation of ducts with lateral and terminal budding as the most advanced change. *Stimulation grade II:* Beginning alveolar growth with a few secretory vacuoles in the small acini. *Stimulation grade III:* Multicentric alveolar growth showing dilated acini lined by mitotically proliferating epithelium and containing abundant secretions. Since within the same mouse the 4 breasts were not always equally stimulated, some overlapping of the findings occurred in the various groups. Hence, in order to obtain quantitative data, the degree of stimulation of each mammary gland was recorded separately, and the findings were tabulated on a percentage basis of the total number of breasts studied at each stage in each experimental group. The results are summarized in Table I.

Results. Ovariectomized non-injected mice: All breasts were resting. The number of ducts in a low power field varied from 4 to 16. The epithelium was low and showed no proliferation. Occasionally small amounts of secretions were seen. No differences in the microscopic appearance could be established in mice of the various strains; nor did the presence or absence of the milk agent alter the microscopic picture.

Strain Z. In mice possessing the agent (Z), growth stimulation of grade II was noted in 4 of 8 breasts (50%) after one week, and in all 8 breasts (100%) after 2 weeks' observation; after treatment for 4 weeks, all 8 breasts (100%) disclosed grade III stimulation (Fig. 1). In mice without the agent (Zb), observed up to 2 weeks, mammary growth did not exceed grade I; after 4 weeks, 4 of 8 breasts (50%) disclosed stimulation grade I, and 3 of 8 breasts (37.5%) showed growth of grade II (Fig. 2). Grade III stimulation was not reached until after six weeks' treatment, and then only 1 of 6 breasts (16.7%) had reached this stage. With prolonged administration of the estrogen, mammary growth became increasingly intensified, and gradually approached conditions seen in the animals possessing the agent. Still, even after 3 months' treatment, the growth response in the breasts of the Zb mice lagged behind that seen in the Z mice.

Strain D2. In mice of this strain, mammary growth was, under the influence of the estrogen, less stimulated than in the Z mice. However, in mice with the agent (D2), the breasts reacted to the estrogen more vigorously than the breasts of mice without the agent (D2b). In the D2 mice, 1 of 8 breasts (12.5%), was resting after 2 weeks of treatment as compared with 6 of 8 breasts (75%) in the D2b mice. Simultaneously, in the D2 mice, 3 of 8 breasts (37.5%) showed grade II stimulation, while in the mice without the agent (D2b) mammary growth did not exceed grade I, and this was seen in 2 of 8 breasts (25%). Grade II stimulation was first noted in 2 of 8 breasts (25%) of the D2b mice after 4 weeks' treatment. After 6 or more weeks of observation, the differences between the growth response in groups D2 and D2b respectively became less distinct. However, none of the mice without the agent developed acini, while of the mice possessing the agent (D2) at least 3 breasts (37.5%) showed grade III stimulation.

Strain A. During the early stages of treatment with the estrogen mammary growth was more accentuated in mice possessing the agent (A) than in those without the agent (Ax). After 2 weeks, 50% of the breasts of the A mice had reached growth of grade II, while those of the Ax mice were not stimulated beyond grade I. After 4 weeks, mammary proliferation of grade II was noted in the Ax group. However, only half as many breasts (37.5%) showed this change as did the corresponding A mice (75%). Again, after 6 weeks, 50% of the breasts of the A mice showed grade III stimulation, while the most advanced growth in the breasts of the Ax mice was still of grade II. After 8 or more weeks, the differences in the growth response of the breasts of the 2 groups began to disappear. In the refostered group (Axa), the reaction of the breasts following the injections of the estrogen was slow. From the second week on, the degree of growth stimulation was intermediate between that of the Ax mice and those who had the agent throughout their entire ancestry (A). The mammary response to estrogen in mice possessing the agent for a varying number of generations may deserve further investigation.

Discussion. Injections of equal amounts of alpha estradiol benzoate into ovariectomized mice of various strains caused more vigorous mammary growth in individuals possessing the milk agent than in mice of the same strains free of the agent. These differences were most conspicuous during the early stages of hormonal stimulation. Thus, the milk agent seems to determine at least partly the response of the breast to estrogen. The failure of previous experiments(3,4) to demonstrate these variations may be due to differences in the methods employed: Whole mount specimens will not reveal the early proliferation of the ductal epithelium, particularly if infolding rather than elongation of ducts occurs. Besides dosage, the time factor may play a role in the mammary response so that investigations of one single stage following administration of the estrogen may not disclose the complete picture. In addition to the differences in the mammary response attributable to the milk agent (strains Z, D2, A, Axa) strain differences in the mammary response to the hormone continued to exist also in the absence of the milk agent. These differences manifest themselves if corresponding stages in strains Zb, D2b, Ax, and C57 black (2a) are compared. These findings are thought to be related to the different genetic constitution of various strains. Strain differences exist in the response of the adrenals and the hypophyses to castration(5-8). The estrogen injected, however, should obviate such castration effects and therewith strain differences in the latter. Apparently then the sensitivity of the target tissue plays a major role in the mammary

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response to hormonal stimulation. The nature of factors determining the tissue susceptibility is under further investigation.

Summary. The mammary glands of ovariectomized mice of strains Z, D2 and A possessing the milk agent were more susceptible to estrogenic stimulation than the breasts of mice of the same strains raised for many generations without the milk agent. However, strain differences in the mammary response to the estrogen continued to exist also in the absence of the milk agent. These variations are considered to be due to strain differences in the susceptibility of the target tissue to hormonal stimulation.

Received June 4, 1951. P.S.E.B.M., 1951, v77.

Effect of Oral 688A (N-Phenoxyisopropyl-N-Benzyl-β-Chloroethylamine Hydrochloride) on Blood Pressure in Normotensive and Hypertensive Subjects.* (18820)

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Previous investigations have shown that Dibenamine, administered intravenously in man, closely approximates the effects of a surgical sympathectomy(1,2). However, its clinical usefulness proved to be of limited value because of its mode of administration and its side effects. These shortcomings of Dibenamine have stimulated the search for an orally effective adrenergic blocking agent, devoid of side effects. A compound, designated 688A, recently developed, seems to meet the requirements for an orally active drug.

In the present report, we wish to summarize the preliminary clinical results obtained with this compound, used since February 1950, in patients with arterial hypertension and peripheral vascular diseases.

Methods and material. 688A (N-phenoxyisopropyl-N-benzyl-β-chloroethylamine hydrochloride) is a new synthetic adrenergic blocking agent.† Chemically it is a derivative of Dibenamine (N,N-dibenzyl-β-chloroethylamine hydrochloride). Pharmacologic studies in animals showed that its adrenergic blocking potency is greater than that of Dibenamine, and that it is active parenterally as well as orally(3,4). 688A was used in enteric coated tablets, supplied in dosages of 10, 20, and 40 mg. The drug was administered 3 times daily with meals. The dosage used varied with the patient and ranged between 30 mg and 560 mg daily. Each patient was first tested carefully for his sensitivity to the drug by starting with a small dose and then increasing it progressively. The optimum daily dose was determined by the therapeutic effectiveness without, or with only minimal, side reactions. Observations were made during treatment with 688A and during administration of placebo. The size and color of the latter were identical to those of the active drug. The subjects were mostly patients admitted to the wards of the Montefiore Hospital.‡ Of a total of 47 subjects used in this investigation,

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* This investigation was supported by a grant from the Smith, Kline and French Laboratories.

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‡ Two patients, included in this series, were hospitalized at the V. A. Hospital, Bronx, N. Y. We are indebted to Dr. Abraham Azulay, Senior Resident in Cardiology, for the data obtained in these two cases with essential hypertension.

19 were normotensives and 28 were hypertensives. Most of these patients were first hospitalized and a control period of observation before and under 688A treatment was obtained under basal conditions. Electrocardiograms, heart size films, eyeground changes and renal functions were recorded before and during treatment. Sodium amytal, intravenous Dibenamine and benzodioxane tests were done on most of the hypertensive subjects. Cold pressor tests were performed in many cases before, and at regular intervals after, starting the treatment. These patients were allowed to be ambulatory. After a minimum of 2 weeks of hospital observation, the patients were discharged and followed twice weekly as outpatient cases. The blood pressure (B.P.) was measured with a mercury manometer with the patient in both the supine and erect positions after he had rested for at least 15 minutes. The pulse rate (P.R.), counted at the wrist, was also recorded in both the supine and erect positions. At least 3 consecutive readings were made for each measurement. Hourly arterial pressure measurements for 6 to 8 consecutive hours were taken during treatment in many of the hospitalized patients. The average values of the daily readings were recorded and the lowest supine B.P. with its corresponding erect B.P. were used for the analysis of the manometric results.

Results. A. *Effect on the blood pressure in the supine and erect positions.* 1. Nineteen normotensive subjects received 688A for periods varying from 2 to 18 weeks. The average control B.P.s for both supine and erect positions were 124/71 and 124/80, respectively. After the drug administration, the average B.P.s for the 2 positions were 107/62 and 96/64 with a mean change of –17/–9 and –28/–16, respectively. 2. In hypertensive subjects, the degree of fall in B.P. varied with the type of hypertension. a. Eight patients with uncomplicated essential hypertension received the drug for periods varying from 1 to 16 months with an average of 6 months. Their average control B.P.s for both supine and erect positions were 177/112 and 175/118. After treatment with 688A the mean B.P.s were 140/91 and 135/95 with a mean change of –37/–21 and –40/–23, respectively. b. Sixteen patients with malignant hypertension were under observation with 688A for periods varying from 2 weeks to 11 months with an average of 3 months. Their average control B.P.s for both supine and erect positions were 221/125 and 220/131. After treatment the mean B.P.s were 182/105 and 155/99 with a mean change of –39/–20 and –65/–32, respectively. c. Three patients with systolic hypertension were under observation with 688A for periods varying from 1 to 4 weeks. Their average control B.P.s for both supine and erect positions were 199/75 and 199/82. After treatment, the average B.P.s were 173/59 and 165/62, with a mean change of –26/–16 and –34/–20, respectively. d. In one case of pheochromocytoma, the control supine B.P. which fluctuated from 153/95 to 240/150 fell to 95/64 after 75 mg of 688A. 3. The daily range of the average dosage of 688A varied from 40 mg to 230 mg for the entire series. The average effective daily dose of the drug varied with the group of patients as follows: 101 mg for normotensives, 130 mg for benign, 194 mg for malignant, and 67 mg for systolic hypertensives. As a rule the maximum action on the B.P. from a single effective dose of 688A was observed 1 hour to 1½ hours after its administration. The effect was noted on both the supine and erect readings, the latter being usually more pronounced. Within 3 to 5 hours, these effects gradually diminished or disappeared.

B. *Effect on the pulse rate.* The P.R. changes following 688A varied with the group of patients. The average changes in the supine position were: + 8 for normotensives, none for benign, –2 for malignant and –1 for systolic hypertensives. The average changes in the erect position were: + 20 for normotensives, + 12 for both benign and malignant, and + 7 for systolic hypertensives.

C. *Effect on symptoms.* Evaluation of symptomatic improvement following any medication in hypertension is notoriously difficult. Effect of placebos and comparison with symptoms before treatment with 688A were used as criteria for this evaluation. Headaches, dizziness, palpitations and dypsnea were common complaints in 22 of the 27 hypertensive subjects. The remaining 5 subjects were completely asymptomatic. The degree of symptomatic improvement in the 22 patients was as follows: good in 9, moderate in 7, poor in 6. The relation between manometric response to the drug and symptomatic improvement was apparent in most cases but not in all.

D. *Other effects.* A total of 51 cold pressor tests performed on 14 subjects before and during treatment usually showed partial inhibition and seldom complete blockade. Time relationship to the administration and dose of the drug appeared to influence the degree of the inhibition to the cold stimuli. Miosis was noted in most cases. Dilatation of the pupil with neosynephrine appeared impossible during drug administration. The results of 688A on the heart size, EKG and fundi were studied in 8 patients with long-term treatment. Heart size changes were not significant, while improvement in both the EKG pattern and fundi were present in 2 patients. Effect of 688A on pain and peripheral blood flow were also studied and will be reported elsewhere.

E. *Side effects.* Side reactions to 688A were few and minor. Dryness of the mouth and stuffiness of the nose were not infrequent. Drowsiness and fatigue or weakness occurred in a few cases and were rather disabling at times. Toxic effects such as nausea and vomiting were rarely noted and were usually accounted for by overdosage. Dizziness and palpitation were observed in several patients especially on rapid change of position. Most of the side effects were observed during the first few days of treatment. With proper adjustment of the dosage the patients displayed little of the above symptoms.

F. *Tolerance to the drug.* The effectiveness of the drug appeared to diminish somewhat in most cases after 2 to 3 months of administration. Increased dosage of the drug became necessary to restore its effectiveness. Tolerance to the drug, however, developed in a few patients receiving 688A over long periods of time. This was studied in 14 patients observed on the drug for periods ranging between 2 and 16 months with an average of 6 months. In 2 cases the B.P. returned to its initial level after 5 and 7 months and was not influenced by further increase in the dosage. In 5 cases tolerance to the drug was partial, while in the remaining 7 cases no tolerance was apparent as long as 11 months later. The effectiveness of the drug varied in the hospitalized and ambulatory patients. The latter usually required higher dosage than the former.

Summary and conclusions. 1. The foregoing results indicate that 688A, a new adrenergic blocking agent, is an effective oral drug. It induces a fall in both supine and erect blood pressures in all subjects. The degree of the manometric effect depends on the dosage used and on the group of patients. The duration of the decrease in B.P. following a single dose is variable and usually lasts 3 to 5 hours. Symptomatic improvement occurred in 16 of 22 patients with hypertension. Improvement was noted in a few cases even in the absence of appreciable fall in B.P. As noted above, evaluation of symptomatic improvement may be difficult, since in a few cases the effects of placebo were equally good, for at least several weeks. Cold pressor tests showed that 688A in the dosage used, usually induced partial inhibition. It is possible that by its specific sympatholytic action 688A, besides its hypotensive effect, also prevents sudden rises in B.P. as indicated by the cold pressor tests. 2. Side effects of this drug were remarkably few and minor. Occasional drowsiness and sensation of fatigue were among the most disabling reactions noted in some patients. Proper dose adjustment seemed to be an important factor in preventing side effects. It should be noted that these side effects actually represent clinical criteria of adequate adrenergic blockade. Toxic symptoms such as nausea, vomiting and possibly fatigue were rarely encountered. Tolerance to the drug was noted in a few cases and may represent a shortcoming in a long-term treatment. 3. While individual responses to the drug were variable, the overall results in the entire group of patients indicate that 688A is effective in lowering B.P. and alleviating hypertensive symptoms. 4. These preliminary results obtained with 688A appear encouraging. The potentialities of this drug in arterial hypertension and peripheral vascular diseases are under further investigation. Detailed studies will be published elsewhere.

The authors wish to express their appreciation to Dr. Louis Leiter, Chief of the Medical Division, and to Dr. Samuel Silbert, Attending Surgeon for Peripheral Vascular Diseases, for their active interest in this investigation.

Received June 4, 1951. P.S.E.B.M., 1951, v77.

Selective Inhibition of Adrenal and Thyroid Stimulating Effect of Amphenone B* by Cortisone and Thyroxine. (18821)

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We have previously reported that the oral administration of Amphenone "B"(1-2) in the rat induces a marked adrenal and thyroid hypertrophy(3). The adrenal stimulation was lacking in the hypophysectomized animal and was prevented by cortisone administration in the intact rat.

This report presents data which show that thyroxine will prevent the Amphenone-induced thyroid hypertrophy without altering the adrenal response. Conversely, cortisone will prevent the adrenal enlargement without materially depressing the thyroid hypertrophy.

Materials and Methods. Female rats of the Holtzman strain were ovariectomized at 24 days of age and were started on these experiments at 57 days of age. A stock pellet ration was fed *ad libitum*. Amphenone B, Cortisone, and Thyroxine were administered daily as indicated in Table I. The animals were autopsied 24 hours after the last injection. The adrenal thyroid and thymus glands were dissected out and weighed to the nearest milligram.

Results and Discussion. Data from a representative series are presented in Table I. It will be seen that when Amphenone "B" is administered for 14 days it induces a marked adrenal and thyroid hypertrophy. The simultaneous administration of thyroxin and Amphenone "B" reduces the thyroid weight from an expected mean of 50 ± 6 to 9 ± 1. However, the adrenal response is not reduced by thyroxine administration. Moreover, the simultaneous administration of cortisone and Amphenone "B" reduces the adrenal weight from an expected mean of 112 ± 18 to 32 ± 8, but the thyroids in this same group of animals remain enlarged. This selective inhibition of the thyrotropic and adrenotropic action by thyroxin and cortisone respectively indicates that the pituitary is capable of simultaneously increasing its thyrotropic activity and reducing its adrenotropic effect or vice versa. It also indicates that there must be two distinct and separate endpoints in the pituitary through which Amphenone "B" exerts its distinctive effects upon the thyroid and adrenal.

The thymus weights indicate that although the adrenal of the Amphenone "B" treated rat is producing some corticoid, its hormone production is not at all in keeping with the increased size of the adrenal. Thus the control thymus weight of 827 ± 62 mg is reduced to 51 ± 11 mg by Amphenone "B" plus cortisone, but only to 408 ± 177 mg by Amphenone "B" alone. This evidence of limited

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[*] 1,2 bis-(p-aminophenyl)-2-methylpropanone-1 dihydrochloride.

[†] National Institutes of Health, Public Health Service, Federal Security Agency.

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TABLE I. Selective Effect of Cortisone and Thyroxine on Adrenal and Thyroid Hypertrophy Induced by Amphenone ''B.''*

Treatment	Final body wt (g)	Adrenal wt (mg)	Thyroid wt (mg)	Thymus wt (mg)
0	230 ± 13	67 ± 13	14 ± 2	827 ± 62
Amphenone B alone	180 ± 21	112 ± 18	50 ± 6	408 ± 177
Amphenone B + cortisone	150 ± 13	32 ± 8	33 ± 8	51 ± 11
Amphenone B + thyroxine	184 ± 26	111 ± 50	9 ± 1	545 ± 177

* Amphenone ''B'' given 32 mg daily *per os* in 0.5 cc H_2O for 14 days. Thyroxine (dl) given 32 μg daily subcut. 0.2 cc of .01 N NaOH for 16 days. Cortisone acetate given 2 mg daily subcut. in 0.2 cc saline suspension for 16 days. Cortisone and thyroxine begun 2 days before Amphenone ''B.'' Each experimental group includes 10 animals.

corticoid production by the enlarged adrenal suggests that Amphenone "B" may be inducing a non-productive hypertrophy in the adrenal similar to that produced by thiouracil in the thyroid(4-5). In any case, it is clear that the availability of such compounds as Amphenone "B" will provide interesting experimental tools for the elucidation of the reciprocal relationship between the anterior pituitary and its various target organs.

Summary. Amphenone "B" induces a marked hypertrophy of both the thyroid and adrenal in the rat. Simultaneous administration of thyroxin and Amphenone "B" prevents the thyroid hypertrophy without reducing the adrenal enlargement. Combined cortisone and Amphenone "B" administration prevents the adrenal enlargement without altering the thyroid hypertrophy. Thus, Amphenone "B" exerts its adrenal and thyroid effect by acting through two distinct end-points in the anterior pituitary.

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Received June 4, 1951. P.S.E.B.M., 1951, v77.

Metabolism of Glycine.[*] (18822)

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The metabolism of glycine in normal mice has been studied after a single intravenous injection of glycine-2-C^{14} by following the specific activity of the uncombined glycine in the plasma, liver and skeletal muscle and by determining the specific activity of the expired CO_2. In this way it has been possible to estimate the size of the body pool of glycine, the turnover time and rate of turnover of body glycine, and the rate of oxidation of the methylene carbon atom of glycine to CO_2. A similar study of glucose metabolism in normal and diabetic rats was reported recently by Feller, *et al.*(1).

Experimental. Glycine-2-C^{14}[†] (1.5-2.0 μg per g body weight), in 0.1 ml of 0.85% saline solution, was injected intravenously into young, adult, Rockland strain white mice. The specific activity of the glycine was 10,000 cpm per μg. The respiratory CO_2 was absorbed in sodium hydroxide solution and then precipitated as $BaCO_3$ for radioactivity meas-

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[*] This work was supported in part by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council.

1. Feller, D. D., Strisower, E. H., and Chaikoff, I. L., *J. Biol. Chem.,* 1950, v187, 571.

[†] Obtained from Oak Ridge National Laboratory on allocation of the U. S. Atomic Energy Commission.

urements. After the appropriate time the animals were sacrificed and deproteinized extracts of plasma, liver and thigh muscles were prepared; the tungstic acid procedure of Schurr, *et al.*(2) was used except that boiling was omitted and the entire process carried out rapidly below 5°. An aliquot of each tissue extract was mixed with phosphate buffer and ninhydrin solution and the mixture was then made up to 8 ml and distilled, as described by Alexander, *et al.*(3). Under these conditions the uncombined glycine is decomposed and the radioactive methylene carbon is converted to formaldehyde which collects in the distillate. The danger of carrying over nonvolatile material which might be radioactive was minimized by using a spray trap and by collecting only the first 6 ml of distillate. In order to determine the formaldehyde concentration in the distillate, a 1.7 ml aliquot was mixed with 0.2 ml of 5% aqueous chromotropic acid solution and the mixture was chilled while the total volume was brought to exactly 3 ml by adding concentrated sulfuric acid. The resulting solution was heated in a boiling water bath for 35 minutes and the optical density was measured at 570 mμ in a Beckman spectrophotometer as recommended by MacFadyen(4). In order to measure the radioactivity of the formaldehyde in the distillate, a 2 ml aliquot was mixed with exactly 1 mg of non-radioactive formaldehyde and the mixture was precipitated by the addition of 10 ml of 0.4% dimedon solution. The mixture was allowed to stand for at least 3 hours and was then filtered onto a paper disc and mounted on a brass holder, substantially as described by Henriques, *et al.*(5). The weight of the sample was determined‡ and the total radioactivity measured was corrected for the difference between the weight of the sample and the theoretical weight of dimedon precipitate from 1 mg of formaldehyde (9.74 mg). The specific activity of the formaldehyde divided by 2.50 gives the specific activity of the uncombined glycine originally present in the tissue extract. All radioactive samples were counted for a time sufficient to bring the statistical error to less than 10 percent and were corrected for self absorption(6).

Results. The specific activity of the carbon in the expired CO_2 and of the uncombined glycine in liver, muscle and plasma are shown in Fig 1. Each point on the liver and muscle curves represents the average of measurements on 3 mice. Each point on the plasma curve represents the average of 2 determinations, each of which was made on pooled plasma from 2 mice. Each point on the expired CO_2 curve represents the average of measurements on 4 mice. The vertical or sloping line through each point indicates the range of the values whose average is represented by the point.

Determination of the size of the body pool of glycine. As shown in Fig. 1, the decrease in the specific activity of the uncombined glycine becomes approximately logarithmic about 15 minutes after the glycine injection. Extrapolation of these curves to zero time as suggested by Feller, *et al.*(1) is considered to indicate what the initial dilution of the labeled glycine would have been if there had been instantaneous mixing of the added glycine with the glycine in the body pool. This interpretation involves the assumption that the dilution of the labeled glycine was going on during the earlier part of the experiment in the same way as during the period of logarithmic decrease in specific activity. In the present experiments this extrapolation indicates that the size of the dynamic body pool of glycine is approximately 30 mg per 100 g body

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2. Schurr, P. E., Thompson, H. T., Henderson, L. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1950, v182, 29.

3. Alexander, B., Landwehr, G., and Seligman, A. M., *J. Biol. Chem.*, 1945, v160, 51.

4. MacFadyen, D. A., *J. Biol. Chem.*, 1945, v158, 107.

5. Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., *Ind. Eng. Chem., Anal. Ed.*, 1946, v18, 349.

‡ Samples were dried at 80°C for 20 minutes. Prolonged heating at higher temperatures results in decomposition and sublimation of sample and decrease in specific activity of residue.

6. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E., *Isotopic carbon,* New York, 1949, pp. 317-318.

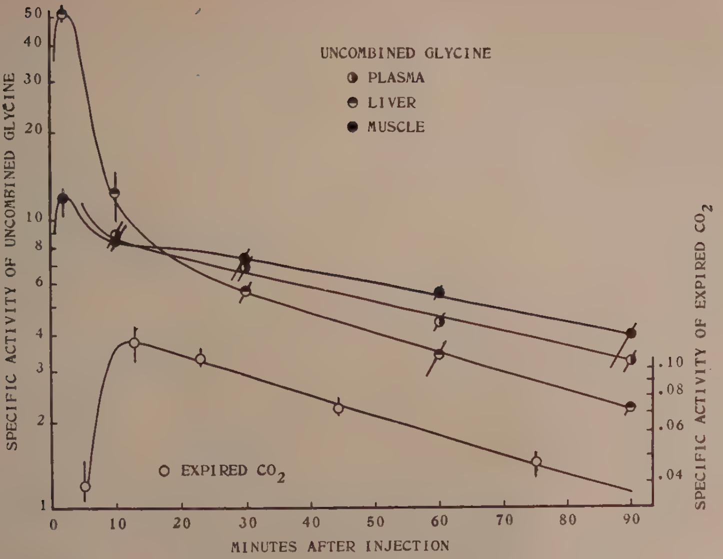

FIG. 1. Specific activity of expired CO_2 and of uncombined glycine in different tissues, following a single intravenous injection of glycine-2-C^{14}. The specific activity is expressed as per-cent of administered dose per mg of glycine or per mg of carbon in the expired CO_2. Each vertical or sloping line indicates the range of values whose avg is represented by the corresponding point.

weight. However, isotope dilution experiments in which a similar quantity of labeled glycine was added to chilled homogenates of whole mice indicated that the amount of uncombined glycine present in the mouse is approximately 10 mg per 100 g. It is evident that within 15 minutes after its injection, the labeled glycine not only mixes with the uncombined glycine present in the mouse but also comes to equilibrium with other compounds. Compounds with which it equilibrates may include the glycine in glutathione (7) and other physiologically active peptides (8), and perhaps serine(9-10). It has been shown by Friedberg and Greenberg(11) and more recently by Borsook, *et al.*(8) that intravenously administered amino acids are removed from the plasma very rapidly. In the present study, the initial rapid rise and the subsequent rapid decrease in the specific activity of the uncombined glycine in the liver indicate that this organ is particularly active in the effort of the organism to dispose of the "extra" glycine which has been injected. It is possible that in this initial readjustment some of the labeled glycine might be irreversibly removed from the glycine pool at a rate which would not be taken into account by the extrapolation to zero time. If this happened, the actual size of the glycine pool would be smaller than that indicated by the extrapolation procedure. In the present case, it is estimated§ that this effect introduces less than 5% error in the pool size.

Turnover time of the glycine pool. During

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7. Waelsch, H., and Rittenberg, D., *J. Biol. Chem.*, 1941, v139, 761.

8. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, 1950, v187, 839.

9. Sakami, W., *J. Biol. Chem.*, 1949, v178, 519.

10. Goldsworthy, P. D., Winnick, T., and Greenberg, D. M., *J. Biol. Chem.*, 1950, v180, 341.

11. Friedberg, F., and Greenberg, D. M., *J. Biol. Chem.*, 1947, v168, 405.

§ If 60 μg of glycine, (the quantity injected in these experiments), was destroyed in the viscera at a time when the specific activity of uncombined glycine was 500 cpm per μg, (higher than that observed in any of these experiments), this would result in the removal of only 30,000 cpm from the glycine pool, *i.e.*, approximately 5% of the 600,000 cpm injected.

the period of logarithmic decrease the times for the specific activity of the glycine to decrease by one-half (t½) are 45, 60 and 75 minutes for liver, plasma and muscle respectively. The mean t½ for the glycine pool is probably close to 1 hour. The time for the turnover of the pool (t_t) is given by the expression $t_t = 1.44$ t½(1) and in this case is approximately 1.5 hours.

Rate of turnover of glycine. Since 30 mg of "glycine" per 100 g is turned over in 1.5 hours, the rate of turnover is approximately 20 mg per 100 g per hour. Except for the most active protein components, the exchange of the labeled glycine with proteins is still in the phase during which glycine of high specific activity is being incorporated and glycine of low specific activity is being liberated. Consequently the turnover rate measured in these experiments includes the exchange of the labeled glycine pool with proteins and is probably close to the actual turnover rate of the glycine pool in the animal. Turnover as used here includes all of the processes by which molecules are removed from the glycine pool and other molecules are added to it. The slopes of the curves in Fig. 1 indicate that the rate of turnover of glycine in liver is much greater than it is in skeletal muscle; moreover, the transfer of glycine between the plasma and the tissues is not quite fast enough to bring the specific activity of the glycine in all parts of the body pool to the same value at a given instant. However, the interchange of glycine between tissues is undoubtedly too rapid to permit the calculation of rates of turnover in individual organs.

Oxidation of glycine to CO_2 The rate of oxidation of glycine to yield CO_2 may be estimated by dividing the amount of radioactivity expired in a given time interval by the mean specific activity of the glycine in the animal during the same time interval, or by the method suggested by Feller, *et al.*(1). Application of either method to the present data indicates that the rate of oxidation of glycine to CO_2 is approximately 3 mg per 100 g per hour. As a result of this oxidation the methylene carbon of glycine contributes approximately 0.2% of the carbon in the respiratory CO_2. It is evident that these mice were synthesizing most of the glycine required in their metabolism. With the semi-synthetic diet used(12) they received in their food not more than 7 mg of glycine per 100 g per day, whereas oxidation at the rate indicated in these experiments would consume approximately 72 mg of glycine per 100 g in 24 hours. This may provide at least a partial explanation for the finding that even 90 minutes after injection of the labeled glycine the specific activity of the uncombined glycine in various tissues did not approach a common value.

Summary. The metabolism of glycine in mice has been studied after a single injection of glycine-2-C^{14} by following the specific activity of the uncombined glycine in plasma, liver and skeletal muscle, as well as the specific activity of the respiratory CO_2. The size of the dynamic pool of glycine was found to be 30 mg per 100 g of which 10 mg per 100 g was uncombined glycine; the turnover time of the glycine pool was 1.5 hours and the rate of turnover 20 mg per 100 g per hour. The amount of glycine oxidized to yield CO_2 in the breath was found to be approximately 10 times the amount of glycine ingested in the food.

The author is indebted to Dr. H. P. Rusch, Dr. R. K. Boutwell and Dr. C. Heidelberger for much helpful advice and to Mrs. Edith Wallestad, Miss Nancy Lake and Mr. A. G. Malman under the supervision of Dr. Heidelberger for assistance in the measurements of radioactivity.

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12. Boutwell, R. K., Brush, M. K., and Rusch, H. P., *Am. J. Physiol.*, 1948, v154, 517.

Received June 4, 1951. P.S.E.B.M., 1951, v77.

Effect of Pertussis, Diphtheria Toxoid and Salmonella Immunization on Experimental Poliomyelitis.* (18823)

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Recent clinical evidence in Australia and England suggests that pertussis vaccine given alone or in combination with diphtheria toxoid during the poliomyelitis season results in a significantly increased frequency and clinical severity of paralysis in the inoculated limb(1-4). No significant difference was noted between diphtheria toxoid injected alone or combined with pertussis vaccine. The great majority of the intervals between the last inoculation and onset of poliomyelitis was less than 30 days. There was no evidence that inoculations carried out 3 months or more before the onset of illness had any such effect. In all of the above reports the usual 1:2 or 1:3 ratio of arm to leg paralysis was reversed in young children who received prophylactic injections predominately in the arms within a 28-day period prior to the onset of the disease. A recent editorial(5) in the *J.A.M.A.* summarizes clinical reports on the relationship between poliomyelitis and prophylactic immunizations.

The present studies were undertaken to study the effect of pertussis vaccine alone or in combination with diphtheria toxoid on the incubation period of Swiss mice inoculated with the Lansing strain of poliomyelitis virus. Experiments showing the effect of vaccinating Lansing virus infected mice with *Salmonella typhimurium* vaccine were also done.

Materials and Methods. Groups of Swiss mice (Plymouth strain) weighing 13 to 15 g were inoculated intracerebrally with approximately 10 LD_{50} units of the Lansing strain of poliomyelitis virus. The identity of the Lansing strain used was verified periodically by neutralization tests with hyperimmune Lansing antiserum. Mice inoculated in each experiment varied from 20 to 80 in number and were equally divided into vaccinated and control animals. The test mice were vaccinated intraperitoneally on the first, third, and fifth days after virus inoculation with 0.5 cc of pertussis vaccine containing 7.5 billion organisms or 0.25 cc combined alum precipitated diphtheria toxoid and pertussis vaccine (3.75 billion organisms). The alum precipitated diphtheria toxoid contained ⅛ of a human immunizing dose. A few experiments were also carried out in which the mice were injected intraperitoneally with 0.25 cc of alum precipitated diphtheria toxoid alone or plain diphtheria toxoid. The alum precipitated toxoid contained ⅛ of a human immunizing dose, while the plain toxoid contained 1/12 of a human immunizing dose. The pertussis vaccines and diphtheria toxoids used were biological products prepared by the Illinois Department of Public Health for human use and were obtained through the courtesy of Dr. H. J. Shaughnessy and Mr. John Neal. The control mice in most experiments were inoculated intraperitoneally (0.5 cc) with nutrient broth after the same intervals.

The *Salmonella typhimurium* vaccine which was used was prepared from a 24 hour culture grown on trypticase agar, killed by heating at 56°C for 1 hour, and adjusted to a suspension containing approximately 1 billion organisms per cc with 0.85% saline solution. The vaccine was preserved with 1:10,000 merthiolate and stored in the refrigerator at 3 to 5°C.

Results. Pertussis Vaccine and Diphtheria Toxoid. Results obtained in a series of 16 experiments carried out by technician A are

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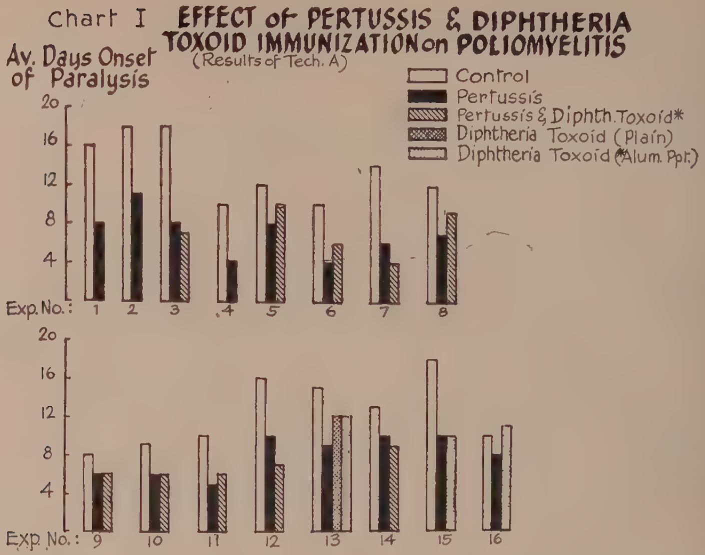
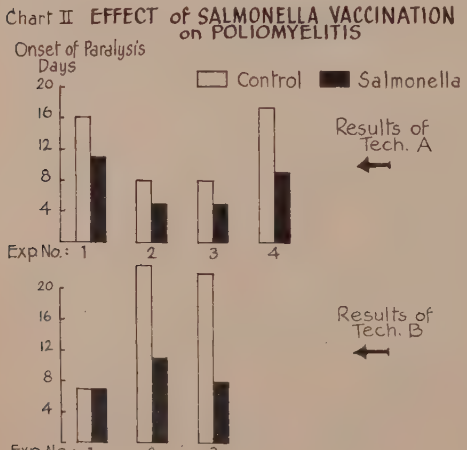
* Supported in part by the Michael Reese Research Foundation. Read before the Society of American Bacteriologists, May 29, 1951, Chicago, Ill.

1. Martin, J. K., *Arch. Dis. Childhood,* 1950, v25, 1.
2. McCloskey, B. P., *Lancet,* 1950, v1, 659.
3. Geffen, D. B., *M. Officer,* 1950, v83, 137.
4. Hill, A. B., and Knowelden, J., *Brit. M. J.,* 1950, v2, 1.
5. Editorial, *J.A.M.A.,* 1950, v144, 240.



given in Chart 1. The chart shows the average incubation period in days after virus inoculation for onset of paralysis in the control and various vaccinated mice. The range of incubation period for both groups varied from 3 to 30 days. Over 500 mice were studied in this series, and there was a minimum of 10 mice in each group of control or vaccinated mice in every experiment. Mice in the first 8 experiments were inoculated with the same pool of Lansing virus, while those in the second group of 8 experiments received a different batch of virus. Chart I also shows the results obtained in identical experiments carried out independently by technician B. Technician B, however, used a different pool of Lansing virus. The graphs in Chart 1 show that a consistent significant reduction in the average incubation period before onset of paralysis was obtained in all experiments in which mice were vaccinated with pertussis vaccine alone or combined with alum precipitated diphtheria toxoid. Statistical analysis of these data was carried out by Dr. Herbert Silverstone using the Student-Fisher t test.[†] According to Dr. Silverstone the differences obtained between the vaccinated mice and controls in all experiments except with the alum precipitated toxoid alone in experiment 16 are significant because the probability value (P) varied from < 0.01 to < 0.001. There was no significant difference between the groups of mice receiving pertussis vaccine alone or the pertussis-diphtheria toxoid mixture. Moreover, there was no sig-



[†] As the variance of each experimental group was proportional to the mean, the statistical tests of significance were conducted with the logarithms of the days of paralysis. The mean values presented are the geometric means. (Bartlett, M. S., *Biometrics*, 1947, v3, 39).

nificant difference in average survival time after onset of paralysis between the control and vaccinated mice in most experiments.

Salmonella Vaccine. We reported(6) that autolyzed brain diluent shortens the incubation period and facilitates the transfer of experimental poliomyelitis. Since then we and others have been unable to reproduce these results. We noted, however, that at the time that we carried out the original experiments with the autolyzed brain diluent the strain of mice (CFW) which we used were infected with *Salmonella typhimurium.* The signs of the infection were readily controlled by feeding the mice sulfadiazine, although the carrier state persisted. It occurred to us that perhaps it was the *Salmonella* rather than the autolyzed brain which was responsible for the enhancing effect, especially since it was found that feeding sulfadiazine to virus inoculated, *Salmonella*-free mice had no significant effect. In order to test this hypothesis, a heat-killed *S. typhimurium* vaccine containing one billion organisms per cc was prepared and injected intraperitoneally (0.5 cc) following virus inoculation in the same manner as described in the pertussis-diphtheria toxoid experiments. The results obtained were too equivocal to be significant although some experiments were highly suggestive.

Recently we have repeated these experiments by inoculating the *Salmonella* vaccine intravenously instead of intraperitoneally. Groups of mice were inoculated intracerebrally with approximately 5 LD_{50} units of Lansing virus. On the first and third days following the virus injection, they were inoculated intravenously with 0.1 cc of *Salmonella* vaccine (100 million organisms). The inoculated mice showed little or no reaction to the *Salmonella* vaccine. Similar numbers of mice were used in the control and vaccinated groups as for the pertussis-diphtheria toxoid experiments. The results obtained by technicians A and B working independently are shown in the graphs of the 7 experiments which were done in Chart II. Except for the results obtained in Exp. 1 by technician B, Dr. Silverstone states that the results are statistically significant in analysis by the t test. The probability value (P) varied from <0.01 to <0.02. In other experiments it was found that *Salmonella* vaccine inoculated intracerebrally (0.03 cc) at the same intervals following virus inoculation had a similar effect in decreasing the incubation period.

Discussion. These experiments indicate that pertussis vaccine given alone or in combination with alum precipitated diphtheria toxoid by intraperitoneal injection after previous intracerebral inoculation with the Lansing strain of poliomyelitis virus significantly decreased the incubation period before onset of paralysis. There was no significant difference between the mice vaccinated with pertussis vaccine or the pertussis-diphtheria toxoid combination. Alum precipitated diphtheria toxoid alone also significantly reduced the interval before onset of paralysis in 2 out of 3 experiments. It was impractical to inject the vaccines in the extremities in these experiments in order to determine the site of onset of paralysis and thereby duplicate the clinical observations. Obviously, a large inoculum would lead to disuse of the limb and confuse the findings. Perhaps the use of larger animals such as the rhesus monkey would successfully overcome this difficulty.

The fact that we were also able to show that heat-killed *Salmonella typhimurium* vaccine injected intravenously after previous intracerebral Lansing virus inoculation also significantly decreased the interval before onset of paralysis in 6 out of 7 experiments may indicate that the effect of vaccination on poliomyelitis is a nonspecific stress stimulus. Possibly the mechanisms involved are similar to those in the so-called "alarm reaction," *i.e.*, the higher incidence and more severe paralysis which occurs in monkeys subject to fatigue and chilling during the incubation period of poliomyelitis(7). We are unable to explain our observation that intraperitoneal inoculation of the *Salmonella* vaccine gave equivocal results, while the intravenous or intracerebral

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6. Milzer, A., and Byrd, Jr., C. L., *Science,* 1947, v105, 71.

7. Levinson, S. O., Milzer, A., and Lewin, P., *Am. J. Hygiene,* 1945, v42, 204.

routes were effective. As already indicated we now believe that it was the *Salmonella* infection in our stock mice rather than the autolyzed brain diluent which was responsible for the apparent enhancing effect of the latter which we previously reported(6).

When the present studies were nearly completed, the paper by Findlay and Howard(8) appeared in which they reported similar findings in mice injected with diphtheria toxoid or pertussis vaccine plus diphtheria toxoid following intracerebral inoculation with the Lansing strain of poliomyelitis virus. Their experiments differed from ours, however, in that all of the vaccines were given intravenously rather than intraperitoneally. They also reported that T.A.B. vaccine given intravenously had a similar effect.

Summary. Swiss mice infected intracerebrally with the Lansing strain of poliomyelitis virus show a significantly decreased incubation period before onset of paralysis following intraperitoneal inoculation with pertussis vaccine, pertussis-diphtheria toxoid or diphtheria toxoid alone. The results obtained were based on a series of 20 experiments carried out by 2 technicians working independently and involving over 650 mice. Similar results were also obtained in 6 out of 7 other experiments in which infected mice were injected intravenously with *Salmonella typhimurium* vaccine. Evidence is presented that *Salmonella typhimurium* is apparently responsible for the previously reported enhancing effect of autolyzed brain tissue diluent.

Addendum. We have found that 30,000 units of procaine penicillin G in peanut oil given intraperitoneally at the same intervals as the pertussis vaccine had no significant effect on the incubation period before onset of paralysis.

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8. Findlay, G. M., and Howard, E. M., *J. Path. and Bact.*, 1950, v62, 371.

Received June 4, 1951. P.S.E.B.M., 1951, v77.

Beneficial Effect of Liver Feeding on Swimming Capacity of Rats in Cold Water.* (18824)

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Considerable data are available indicating that in addition to the known nutrients substances are present in our diet which may be required in increased amounts during conditions of stress. Such factors are apparently dispensable under normal conditions, or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to such an extent that deficiencies occur, manifested by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient (1,2). Whole liver is a potent source of such unknown nutrients. In the present communication data are presented which indicate that whole liver contains a factor, apparently distinct from any of the known B vitamins, which significantly increased the capacity of rats to withstand the stress of swimming in cold water.

Procedure. The basal ration employed in the present experiment consisted of sucrose,

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* This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, has been assigned number 341 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

1. Ershoff, B. H., *Physiol. Rev.*, 1948, v28, 107.

2. Ershoff, B. H., Nutrition Fronts in Public Health, 1951. The National Vitamin Foundation, Inc., New York.

61%; casein,[†] 24%; salt mixture,[‡] 5%; cottonseed oil (Wesson), 10%, and the following synthetic vitamins per kg of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; ascorbic acid, 200 mg; 2-methyl-naphthoquinone, 10 mg; and choline chloride, 2 g. To each kg of diet were also added 8000 U.S.P. units of vit. A[§] and 800 U.S.P. units of vit. D.[‖] The vitamins were added in place of an equal amount of sucrose. Each rat also received once weekly a supplement of 4.5 mg alphatocopherol acetate. In addition to the basal ration the following diets were also employed: (1) basal ration plus the following additional vitamin supplements per kg of diet: thiamine hydrochloride, 20 mg; riboflavin, 20 mg; pyridoxine hydrochloride, 20 mg; calcium pantothenate, 60 mg; nicotinic acid, 60 mg; biotin, 5 mg; folic acid, 10 mg; p-aminobenzoic acid, 400 mg; inositol, 800 mg; and vit. B_{12}, 150 μg; and (2) basal ration plus 10% whole liver powder.[¶] The vitamin supplements and the whole liver powder were added in place of an equal amount of sucrose. Sixty female rats of the Long-Evans strain were selected for the present experiment at 22 to 25 days of age and an average weight of 44.2 g. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed the above diets *ad lib.* (20 animals per group). Diets were made up weekly and stored under refrigeration when not in use. Animals were fed on alternate days. All food not consumed 48 hours after feeding was discarded. These measures were employed to minimize oxidative changes in the diet. After 12 weeks of feeding the following body weights were obtained for rats in the various dietary groups: Basal ration, 215.4 ± 6.8 g;[**] basal ration plus B vitamins, 228.8 ± 9.0 g;[**] and basal ration plus 10% whole liver, 247.7 ± 6.9 g.[**] Subsequent to this period swimming tests were conducted on all animals. The procedure employed was as follows: Rats were placed in a barrel approximately 33 inches in height and 28 inches in maximum diameter, with smooth vertical sides, and filled to a depth of 18 inches with water. Tests were conducted at a water temperature of both 36°C and 20°C. Eight rats from each dietary group were tested at the higher temperature; the remainder (12 animals per group) at the lower. Measurements were made of the length of time that rats would swim before remaining submerged for a period of 15 seconds.

The selection of the end point was based on preliminary studies in which it was observed that rats almost never remained submerged below the surface of the water for more than 5 seconds and almost never for more than 10 seconds of their own accord. As the end point of a swim was approached, the rat would submerge for periods of 5 to 10 seconds with increasing frequency and could reach the surface only with obvious difficulty. Of 30 rats tested only 2 rose to the surface after remaining submerged for a period of 15 seconds; and these swam for less than 50 seconds before submerging again, at which time they drowned. Rats remaining below the surface for more than 15 seconds almost invariably drowned.

Results. No significant difference was observed in the swimming performance of rats on the various dietary regimes at a water temperature of 36°C. All of the rats in each dietary group survived a test period of 120 minutes at the end of which they were still swimming. When tests were conducted at a water temperature of 20°C, however, a significant difference was observed between the various dietary groups. Rats which had been fed the basal ration swam an average of 13.3

[†] Vitamin Test Casein, General Biochemicals, Chagrin Falls, O.

[‡] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Chagrin Falls, O.

[§] MYVA-DRY Powder, Distillation Products, Rochester, N. Y.

[‖] HY-DEE Powder, Standard Brands, New York.

[¶] Desiccated Liver, Armour & Co., Chicago, Ill.

[**] Including standard error of the mean calculated as follows: $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

minutes (range 5 to 29 minutes); those which received the basal ration plus B vitamin diet swam 13.4 minutes (range 10 to 23 minutes); only 3 of 12 rats tested in the liver group, however, swam for less than 2 hours (63, 83 and 87 minutes); all other rats in this group were still swimming at the end of 120 minutes at which time the test was discontinued.

No data are available to indicate what factors were responsible for the improved swimming performance of rats on the liver ration at a water temperature of 20°C. Differences in the rate or degree of heat loss between the various groups while swimming in water at a temperature of 20°C did not appear to be responsible for the diverse effects. In this series the body temperature of rats (as determined rectally in a subsequent experiment) rapidly fell from an initial value of 37°-37.5°C to 23°-25°C. The rate of fall was approximately 1°C per minute for rats fed the basal ration or the basal ration plus vitamin diet and somewhat less (about 0.6°-0.7°C per minute) for rats in the liver group. After 30 minutes, however, the body temperature of rats fed the liver-containing diet was as low or lower than that of rats in other groups; nevertheless, these animals continued to swim for a prolonged period of time. At a water temperature of 36°C no significant changes in body temperature occurred on any of the diets employed.

Swimming tests were repeated on the 4th and 7th day after the initial test on all rats under conditions similar to those first employed. No improvement resulted in successive tests in the swimming performance of rats in the 20°C series on either the basal ration or basal ration plus B vitamin diet. The average swimming time of rats in these groups for the 2nd and 3rd test did not differ by more than 3 minutes from that obtained in the initial test. Similarly no significant differences occurred in other groups between the first and subsequent tests.

Swimming tests were also conducted with rats similar in age and weight to those employed above but which had been raised from weaning on a natural food ration.[††] At a water temperature of 36°C all rats tested (6 animals) swam for 120 minutes at which time the test was discontinued. At a water temperature of 20°C, the average swimming time of 8 rats was 24.7 minutes (range 15 to 39 minutes). The average swimming time of rats fed the natural food ration was approximately twice as long at a water temperature of 20°C as that of comparable animals fed the basal ration or basal ration plus B vitamin diet; it was still significantly less, however, than that of rats fed the liver-containing ration. It would appear, therefore, that such protective factors as may have been present in the natural food ration were not present in sufficient amounts for an optimal effect.

Experiments similar to the above were also conducted with young rats. Forty-eight female rats of the Long-Evans strain were selected at 21 to 23 days of age and an average body weight of 42.4 g and were fed *ad lib.* the 3 experimental diets employed above (16 animals per group). After 24 days of feeding the average body weight of rats in the various groups was as follows: Basal ration, 138.1 g; basal ration plus B vitamins, 146.4 g; and basal ration plus whole liver, 149.2 g. Swimming tests were conducted at this time on all rats. At a water temperature of 36°C, all rats tested (6 animals in each dietary group) swam for 120 minutes at which time the test was discontinued. At a water temperature of 20°C, rats fed the basal ration swam an average of 9.8 minutes (range 6 to 13 minutes); those on the basal ration plus B vitamin diet an average of 10.7 minutes (range 7 to 15 minutes); and those on the liver-containing ration an average of 37.4 minutes (range 9 to 155 minutes) (10 rats per group). The beneficial effect of liver on the swimming capacity of rats at a water temperature of 20°C was thus evident in young animals as well as adults. Three of the rats in the liver group, however, swam for less than 15 minutes and did not differ significantly in swimming capacity from animals on the basal ration or basal plus B vitamin diet. It would appear, therefore, that more than 24 days of feeding is required, under conditions of the present experiment, for liver to exert its optimal effect.

[††] Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.

Subsequent experiments were conducted in an effort to determine whether the greater swimming capacity of rats on the liver ration at a water temperature of 20°C was due to an increased production of adrenal cortical hormone(s). Eighteen female rats of the Long-Evans strain were selected at 23 to 25 days of age and fed *ad lib.* the basal ration plus B vitamin diet employed above. After 12 weeks of feeding the animals were divided into 3 groups and swimming tests were conducted at a water temperature of 20°C (6 animals per group). Each rat in group I received an intraperitoneal injection of 5 mg ACTH‡‡ 30 minutes before the test; each rat in group II received an intraperitoneal injection of 5 mg cortisone acetate§§ seven minutes before the test; rats in group III served as untreated controls. No significant difference was observed in the swimming performance of rats in the various groups. The average swimming time of animals in group I was 11.8 minutes; in groups II, 11.4 minutes; and in group III, 12.6 minutes. It would appear, therefore, that an increased production of ACTH or cortisone in response to stress was not the cause of the greater swimming capacity of rats fed the liver-containing diet at a water temperature of 20°C.

Discussion. Findings indicate that immature rats raised to maturity on a purified ration containing 10% whole liver powder swam for a significantly longer time at a water temperature of 20°C than rats fed a similar ration in which the B vitamins were provided in synthetic form. The beneficial effect of liver was apparently not due to its content of known B vitamins. Available data do not indicate, however, whether its protective effect was due to an unidentified factor or to its content of known nutrients. In view of the fact that military personnel and others may on occasion be forced to spend prolonged periods of time in water at a temperature of 20°C or below, it would appear that the identification of a factor which might be employed to increase resistance to this form of stress may have considerable practical value.

Summary. Immature rats raised to maturity on a purified ration containing 10% whole liver powder swam for a significantly longer period at a water temperature of 20°C than rats fed a similar ration containing the B vitamins in synthetic form. The protective factor in liver was distinct from any of the known B vitamins.

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‡‡ ACTHAR, Armour and Co., Chicago, Ill. The material employed was diluted with saline solution to a concentration equivalent to 5 mg of the standard LA-1-A per cc.

§§ Saline Suspension of Cortone Acetate, Merck and Co., Rahway, N. J. Each cc contained 25 mg of cortisone acetate.

Received June 4, 1951. P.S.E.B.M., 1951, v77

Effect of ACTH on Whole Blood Coagulability.* (18825)

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Administration of ACTH in sufficient dosage induces numerous metabolic changes. Reports of the effect of this hormone on the coagulation time of whole blood, however, have been conflicting. Cosgriff *et al.* conclude that ACTH shortens the clotting time, induces hypercoagulability and a state conducive to thromboembolic complications(1). Smith *et al.*, on the other hand, reported a

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* The author is indebted to Drs. Albert E. Renold and Peter H. Forsham for continued interest in this study, and to Dr. H. Cluxton, of the Armour Laboratories, Chicago, Ill., for the ACTH used in this work.

1. Cosgriff, S. W., Diefenbach, A. F., and Vogt, W., *Am. J. Med.*, 1950, v9, 752.

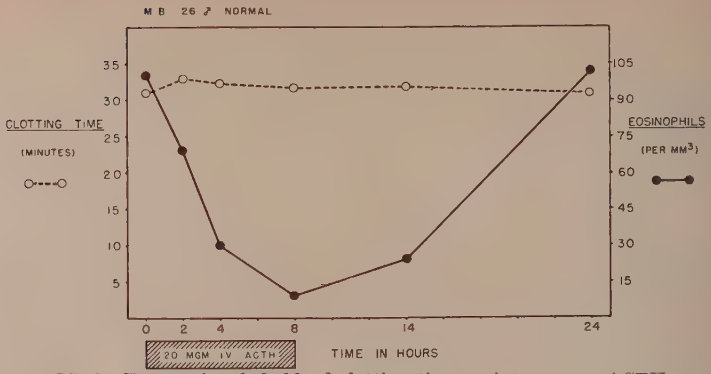

FIG. 1. Changes in whole blood clotting time on intravenous ACTH.

prolongation of the clotting time(2). Although these authors showed an increase in circulating heparin-like factors following ACTH administration, no change in protamine titration was found by the other group. Neither could find any significant alteration in 2-stage prothrombin values. Ac-globulin, prothrombin time or anti-fibrinolysin. These previous reports conflict primarily on interpretation of changes in the whole blood clotting time. This apparently simple test can be easily misleading. In the present experiments a carefully controlled and sensitive, modified Lee-White test of whole blood coagulability was applied in order to evaluate possible changes induced by ACTH administration.

Methods. The clotting time of whole blood was determined by a technic modified from that of Allen(3). Venepuncture is made with a No. 20 (or larger bore) needle and a dry syringe with minimal blood stasis. After releasing the tourniquet, at least 2 ml of blood is drawn into the initial syringe. This syringe is then removed from the needle and an oiled syringe, in which the "dead" space is filled with mineral oil, is substituted. Five ml or more of blood is then withdrawn gently, without difficulty and without air bubbles. Timing begins when blood enters this syringe. When the desired amount is obtained, the needle is quickly withdrawn from the arm and removed from the syringe and one ml of blood is run carefully into each of 5 test tubes. Ten minutes after blood withdrawal, the first tube is tilted horizontally and this is repeated at intervals of one minute thereafter until at least half of the blood is clotted, as observed by giving the tube a sharp snap through a 90 degree arc, when this becomes necessary. When the first tube has reached this end point the time is noted and the second tube is tilted one minute later and at similar intervals until its blood clots. This procedure is repeated until the end points for all tubes have been obtained. The clotting time recorded for the test is the average of the time recorded for tubes 4 and 5, the last 2 tubes. The normal range was 25-40 minutes, usually 30-35 minutes, by this method. Variations in clotting time done at the same time in 2 different sets of tubes did not exceed 3 minutes or 10% of the average value obtained. It should be noted that the test tubes measure 100 x 13 mm and are scrupulously cleaned with scrubbing and acid chromate before being repeatedly rinsed with distilled water. All tests in this study were carried out by the same person. The possibility existed that mineral oil in the second syringe might alter the contacting blood. However, when parallel experiments were carried out by taking blood directly into a third siliconed syringe, after removing sufficient blood in the oiled syringe, no difference in end point was observed between blood collected in either syringe. Blood collected each

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2. Smith, R. W., Margulies, R. R., Brennan, M. J., and Monto, R. W., *Science*, 1950, v112, 295.

3. Allen, J. G., *Transactions of the Third Josiah Macy, Jr., Foundation Conference on Blood Clotting and Allied Problems,* New York, 1950, p. 213.

TABLE I. Short-Term ACTH Effect on Whole Blood Coagulation Time.

Patient	Dose, mg	Route	Control	Coagulation time (min)				
				0-4 hr after end of intravenous infusion	hr after beginning intramuscular ACTH test			
					4	24	48	64
H.	20	I.V.	32	33				
B.	20	I.V.	35	34				
M.	20	I.V.	34	35				
F.	20	I.V.	35	34				
A.	20	I.V.	36	36				
P.	20	I.V.	39	39				
M.	55	I.M.	33		34	32	35	35
A.L.	55	I.M.	36			37	37	36
B.	55	I.M.	35		37			

way was also added to siliconed test tubes, but the normal clotting time in the silicone tubes was in the range of 2 hours and was deemed impractical for the present work.

Experimental. The possible effects of ACTH on the whole blood coagulation time were studied from the standpoint of (a) acute, short-term administration, (b) prolonged therapy, (c) withdrawal, (d) natural or artificially induced hypo- and hyper-adrenal states. Two studies with cortisone are included. ACTH was administered in 20 mg doses in 500 ml solutions by means of an intravenous infusion over an eight hour period (4). When the intramuscular route was used, 25 mg, followed by 10 mg every 6 hours, was given.

Intravenous ACTH administration to a normal subject caused an eosinophil fall to less than 5% of the control value, while the coagulation time remained unchanged (Fig. 1). Similarly, in 6 patients (Table I) no change in the coagulation time was observed at the end of the 8-hour administration period or within the following 4 hours, when maximal metabolic effects are present During intramuscular ACTH administration the coagulation time was determined at 0, 4, 24 and 48 hours and no significant change was observed.

Four patients were studied during 2-3 week periods of therapy. The findings are charted in Table II. No alteration or trend was noted during prolonged ACTH administration. It should be emphasized that these patients showed evidence of maximal adrenal stimulation, as evidenced both by clinical response and by increase in the urinary 17-ketosteroid excretion. These patients were also followed after cessation of therapy (Table II) with an inconsistent suggestion of prolonged clotting. This was not considered significant.

Studies were done in pathologic conditions which are characterized by abnormal adrenal cortical activity. Neither a patient with Addison's disease nor two patients maintained on cortisone following bilateral adrenalectomy showed unusual coagulation time (Table III). Patients with natural and induced Cushing's syndrome were also without abnormalities in the whole blood clotting time.

Two patients treated with large doses of cortisone, given by mouth and by intramuscular injection, showed no coagulation changes (Table II).

Discussion. In contrast to 2 previous but contradictory reports, the present investigation showed no significant change in the whole blood coagulation times in normal subjects and in patients receiving ACTH. Two differences contrast this and the previous reports. The intramuscular route of administration was utilized in the earlier work, while the intravenous route was primarily adopted in this study. However, the intramuscular technic was used in a few cases and no differences were observed. Also, the intravenous route, while using less total ACTH, has been demonstrated to be a more effective way of stimulating the adrenal cortex(4).

Further, the methods of determining the coagulation time in the previous investigations are somewhat different from ours. The value

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4. Renold, A. E., Forsham, P. H., Maisterrena, J., and Thorn, G. W., *New Eng. J. Med.*, 1951, v244, 796.

TABLE II. Long-Term Effect of ACTH and Cortisone on Whole Blood Coagulation Time.

Patient	Sex	Diagnosis	Daily dosage (mg)	Drug route	Control	Day of therapy 1	2-4	5-7	8-10	11-15	16-21	Day after therapy 1	2	3	4-9	10-15
F.	M	Spinal cord tumor	20	A* I.V.	35	36		36	31	33		34	38		36	
M.	M	Rheumatoid arthritis	20	A I.V.	35	35				34	31	33		38	43	39
J.	F	Acute rheumatic fever	20	A I.V.	36					34		34		35	36	
G.	M	”	20	A I.V.	38	40			31		33					
J.	F	”	400	C* P.O.	33	34	31		33							
B.	F	Rheumatoid arthritis	500	C I.M.	32				33	30	30					

* A = ACTH. C = cortisone.

TABLE III. Whole Blood Coagulation Time in Abnormal Adrenal States.

Patient	Diagnosis	Clotting time (min)
C.	Addison's disease	34
H.	100 mg cortisone/day; bilat. adrenalectomy, 52 days postoperative	34
K.	25 mg cortisone/day; bilat. adrenalectomy, 75 days postoperative	35
A.	Cushing's syndrome	30
M.	After 10 mo continuous ACTH; dermatomyositis; Cushingoid syndrome	37

and pitfalls of the whole blood coagulation time determination have been discussed in some detail elsewhere(5-7). The need for elimination of tissue juice contamination should be especially emphasized. In 3 instances during this study blood did not flow freely into the second syringe and there was some question as to whether the open end of the needle was completely within the vein, having either incompletely entered or pierced it. Clotting times in each instance were less than 22 minutes and immediate repetition of the test using a good draw from another vein gave clotting times well within the normal range. Bubbles and dirty glassware may also accelerate the coagulation process. Oily test tubes may prolong clotting.

Summary. A reproducible sensitive test, which minimizes contamination with thromboplastically active tissue juices is outlined for determination of the whole blood coagulation time. No significant alteration of the coagulation time was found with the administration of ACTH to human subjects or in patients with hypo- or hyper-adrenocorticism when this method was used.

Received June 7, 1951. P.S.E.B.M., 1951, v77.

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5. Lee, R. I., and White, P. D., *Am. J. Med. Sci.*, 1913, v145, 495.

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7. Barker, N. W., and Margulies, H., *Transactions of the Second Josiah Macy, Jr., Conference on Blood Clotting and Allied Problems*, New York, 1949, p. 106.

Studies on Estrogen Tri-*p*-anisylchloroethylene. (18826)

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Laboratory studies have demonstrated that tri-*p*-anisylchloroethylene (Tace*), like other estrogens, causes estrogenic changes in the mammary gland, uterus, and vagina, sensitizes the uterus to progesterone, produces abortions

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* Trademark of The Wm. S. Merrell Co. Tace was prepared by M. G. Van Campen in the Organic Research Laboratories of this Company.

TABLE I. Duration of Estrogenic Activity in Castrate Female Rats. Periods shown are the times at which one-half of the animals no longer exhibited an estrogenic vaginal smear. 18 animals in each series.

Route	Compound	Dose		Median duration, days
		mg	R.U.	
Oral	Tace	1	47	2
		5	238	17
	Hexestrol	1	83	3
		5	415	2
	Diethylstilbestrol	1	333	2
		5	1666	3
Subcutaneous	Tace	1	18	53
	Hexestrol	1	2857	2
	Diethylstilbestrol	1	2500	3

in rats, does not alter thyroid abnormalities from thiourea administration and inhibits the gonadotropic hormone production by the pituitary of castrate female rats in parabiosis.

Preliminary experiments(1) in these laboratories indicated there are important differences between the pharmacology of Tace and the better known estrogens.

Experimental. Duration of action in rats. Data in Table I demonstrate that Tace is long acting following either large oral doses or comparatively small subcutaneous doses and short acting in small oral doses. Hexestrol and diethylstilbestrol are short acting in large or small oral and subcutaneous doses.

Duration of action in monkeys. Results of typical experiments in monkeys (Fig. 1) demonstrate that oral doses of 7.0 mg of Tace and 35 mg of hexestrol produce similar changes in vaginal smears, and uterine bleeding occurs at approximately the same time. A single large oral dose of 70 mg of Tace is long acting. The vaginal changes during the first 15 days are similar to those produced by the 7.0 mg dose of Tace. On the fifteenth day the vaginal cytology is typical of the castrate animal. However, without additional dosing, another period of estrogenic activity commences and the vaginal cytology remains indicative of estrogenic activity until approximately the thirty-fifth day.

Elimination. Results of preliminary work in monkeys and the data for a complete experiment in one monkey and in rats (Table II) demonstrate animals administered hexestrol orally excrete only small amounts of estrogenic material. Elimination is primarily in the urine and is completed within three days. Large amounts of estrogenic material are recovered from the excreta of the monkey administered Tace orally. The greater part is eliminated in the feces during the first 3 days, but considerable amounts are recovered up to 30 days after a single oral dose. Similarly in rats, fecal elimination of estrogenic material is high during the first day, but considerable amounts of estrogenic material are recovered as long as 10 days after oral administration. The recovery of estrogenic material equivalent in potency to considerably more than the activity of the administered dose of Tace means that the excreted estrogen must be a metabolic product of greater potency than the parent compound. This is indicated also by the fact that the estrogen in the feces is short acting. This was demonstrated by administering the fecal estrogen orally to 20 castrate female rats in doses equivalent to 5.0 mg of Tace. The duration of action was 4 days as compared to 21 days for an equivalent dose of Tace. It is conceivable that the estrogen eliminated in the feces during the first few days following oral administration of Tace might have been unabsorbed material. How-

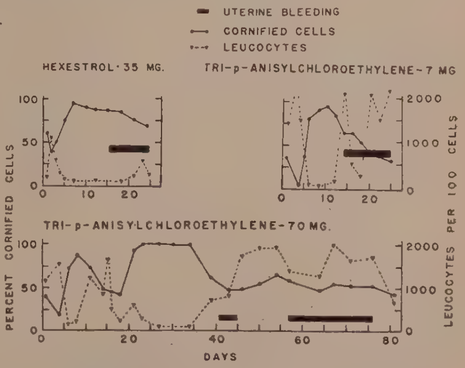

FIG. 1. Responses of castrate female monkeys to single oral doses of Tace (tri-p-anisylchloroethylene) and hexestrol.

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2. Evans, J. S., Hines, L., Varney, R., and Koch, F. C., *Endocrinology*, 1940, v26, 1005.

TABLE II. Estrogenic Content of Ether Extracts of Excreta of Castrate Female Monkeys and Rats Determined by the Method of Evans, *et al.*(2).

		Estrogen recovery expressed as % of dose of estrogen administered					
Days after estrogen adminis.		Tace		Hexestrol		Control	
		Urine	Feces	Urine	Feces	Urine	Feces
Monkeys*	1	8.4	231	.5	.2	0	0
	2	8.7	200	.2	0	0	0
	3	4.3	30	.1	0		
	4	1.2		0			
	5-7	.9	18	0	0		
	8-10	.6	6				
	11-14	.4	2				
	15-29		6				
	30-36		1.4				
	Total	24.5	494.4	.8	.2		
Rat†	1	.16	110	.2	0	0	0
	5	0	2	0	0	0	0
	10	0	2.7	0	0	0	0

* One monkey received single oral dose of 70 mg of Tace and one monkey single oral dose of 35 mg of hexestrol.

† Avg values for dose groups of 4 rats administered three 5 mg oral doses of Tace and hexestrol in one day.

TABLE III. Avg Estrogenic Concentration in Body Fat of Groups of 4 Castrate Female Rats Administered Three 5 mg Oral Doses of Tace and Hexestrol in One Day. Values are biologic equivalent of estrogen administered determined by the method of Evans, *et al.*(2).

Day following last dose	Mg of estrogen per 100 g fat		
	Tace	Hexestrol	Control
1	11.4	0	0
5	5.9	0	0
10	2	0	0

ever, considerable amounts of estrogenic material must have been secreted into the gastrointestinal tract because (1) estrogenic material is eliminated in the feces for thirty days or more following a single oral dose, (2) the eliminated material is short acting orally unlike Tace itself, (3) estrogenic material equivalent to 20.8% of the administered dose of Tace is recovered from feces of 4 rats during a 24-hour period following a single intraperitoneal administration.

Fat storage. The prolonged effect of Tace following oral administration suggests tissue storage. Since Robson and Ansari(3) reported that a,a-bis(p-ethoxyphenyl)-β-phenylbromoethylene was stored primarily in body fat, fat storage was studied following Tace administration. Considerable amounts of estrogenic material are stored in the body fat of the rats which receive Tace (Table III), and the fat concentration gradually decreases over a period of more than 10 days. Hexestrol administration is not followed by storage of estrogenic material in body fat.

Repeated administration. Doses of 5 mg of Tace and 0.1 mg of hexestrol were administered subcutaneously 3 times a week for 13 weeks to groups of 40 normal female rats. A complete report on chronic effects will be made elsewhere. Both estrogens similarly depress the rate of increase in body weight. Uterine and vaginal changes are similar for the two compounds and resemble those reported in the literature for other estrogens. Both substances cause lobule-alveolar growth and evidence of secretory activity of the mammary glands. In general, the mammary gland hyperplasia is more pronounced in animals administered hexestrol than in those administered Tace.

Average weights of pituitaries, adrenals, and ovaries are presented in Table IV. Tace decreases and hexestrol increases the weight of the pituitary. Tace causes a slight increase and hexestrol a pronounced increase in weight of the adrenals. Tace administration results in less ovarian atrophy than hexestrol.

Liver glycogen values (Table V) for rats fed *ad libitum* until sacrificing are not significantly different for the control, Tace, and hexestrol groups. The glycogen content of

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3. Robson, J. M., and Ansari, M. Y., *J. Pharm. and Exp. Therap.*, 1943, v79, 340.

TABLE IV. Avg Organ Wt in mg and Standard Errors for Female Rats Administered Repeated Doses of Tace and Hexestrol.

		Adrenal		Ovary	
Group	Pituitary	Left	Right	Left	Right
Tace	8.4 ± .69	25.2 ± 1.26	24.8 ± 1.12	15.2 ± .74	15.1 ± .82
Hexestrol	26.6 ± 1.32	38.4 ± 1.45	37.3 ± 1.33	8.7 ± .46	8.8 ± .60
Control	11.4 ± 1.16	22.1 ± .60	20.2 ± .61	26.1 ± 1.26	26.1 ± 1.41

TABLE V. Glycogen Content of Livers of Female Rats Administered Repeated Doses of Tace and Hexestrol.

	g glucose per 100 g wet tissue and standard errors	
Compound	Fed *ad libitum*	Food withheld 24 hr
Tace	3.5 ± .20	.2 ± .04
Hexestrol	3.1 ± .16	1 ± .24
Control	3 ± .21	0 ± .01

livers of rats receiving no food 24 hours prior to sacrificing are considerably greater for the hexestrol than for the Tace and control groups. Liver glycogen in the fasted group administered Tace is low but significantly greater than the value for the control group. This difference in glycogenetic activity is related possibly to a greater effect of hexestrol on the adrenals.

Because of temporal differences in the action of Tace and hexestrol, strictly comparable physiologic doses cannot be defined. The following evidence suggests, however, that the differences reported for repeated administrations probably represent differences in activity rather than dosage effects: (1) the doses of Tace employed in the chronic experiment were very large, and it is well established that even small doses of the natural and better known synthetic estrogens cause pituitary enlargement; (2) the two estrogens had similar effects on the weight curves and direct estrogen target organs, but Tace had a lesser effect on organs in which the action is mediated through the pituitary.

Gardner(4) also found that Tace differed from α-estradiol benzoate with repeated administration to CC_1 hybrid mice. Animals receiving Tace developed interstitial cell tumors of the testes while animals receiving α-estradiol benzoate did not. He indicated this difference may be related to either a longer survival time of Tace-treated animals or to a more marked effect, directly or indirectly, on the testes.

Summary. The pharmacology of Tace (tri-p-anisylchloroethylene), a new compound with estrogenic activity, differs in important respects from that of hexestrol which is typical of the natural and better known synthetic estrogens: (1) Tace does not cause and hexestrol does cause pituitary enlargement in rats; (2) Tace has a lesser but similar effect to hexestrol on organs in which the action of the two compounds is mediated through the pituitary; (3) fat storage resulting in long activity occurs following oral administration of large doses of Tace, while there is no fat storage and action is short following hexestrol administration; (4) estrogenic material equivalent in biologic potency to more than the administered dose of Tace is eliminated with the feces, while only small amounts of estrogenic material are eliminated following hexestrol administration.

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4. Gardner, W. U., and Boddaert, J., *Arch. Path.*, 1950, v50, 750.

Received June 15, 1951. P.S.E.B.M., 1951. v77

A Specific Soluble Substance Involved in Nephrotoxic Nephritis.* (18827)

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The experimental nephritis induced in rats by the injection of anti-rat kidney serum has been studied intensively since it was first described by Masugi(1). Smadel(2) obtained anti-rat kidney serum by repeated intraperitoneal injections into rabbits of suspensions of perfused rat kidney. The anti-rat kidney serum when injected intravenously into rats, produced either fulminating or chronic nephritis, depending upon the dosage. This nephrotoxic nephritis was well characterized by Smadel(2,4) and Smadel and Farr(3). Little information has been obtained, however, as to the exact nature of the kidney antigen which gives rise to nephrotoxic antibodies. Swift and Smadel(5) found that when a suspension of ground-up kidney was placed in the refrigerator for 2 days and centrifuged for one-half hour at 2,500 r.p.m. some antigen occurred in a layer of suspended particulate matter overlying a bottom layer of the more solid material. Two investigators have presented evidence that the antigen is located in the glomeruli and not in other portions of the kidney: Solomon *et al.*(6), by separating kidney into glomerular and tubular fractions, demonstrated that only the glomeruli could absorb the nephrotoxic rabbit serum globulin; Pressman *et al.*(7), in experiments that employed radioactive anti-mouse kidney serum, demonstrated localization of radioactivity in the glomeruli and showed that the anti-kidney serum is largely organ-specific. Eisen *et al.*(8) found that the kidney antigen could not be dissolved by a variety of solvents and noted its resistance to heat. Loeb *et al.*(9) produced nephritis in the rat by the injection of anti-rat placenta serum which had been developed in rabbits.

It is the purpose of this communication to report that a substance has been obtained in solution from rat kidney which will combine with the nephrotoxic antibodies and render anti-kidney serum harmless to the rat. This material has not previously been obtained in solution. Some of the chemical properties of this material have been characterized. The substance was assayed by absorption in anti-kidney serum which was then injected into rats. When no nephritis ensued, it was believed that the nephrotoxic antibody in the serum had combined with a specific soluble substance. The fact that it was obtained in a soluble form greatly increases the possibility of further characterization of the substance which is responsible for the phenomenon of nephrotoxic nephritis.

Methods and materials. I. Preparation of anti-rat kidney serum. Wistar rats weighing 200 to 300 g were sacrificed and perfused with 2 liters of 0.85% sodium chloride solution. The perfusate entered through a cannula inserted in the thoracic aorta and escaped by way of an incision in the vena cava. The kidneys were frozen and stored. In preparation for injection, kidneys were ground in a mortar under aseptic conditions and injected intraperitoneally into rabbits following the schedule of Smadel(2). Pooled rabbit serum was distributed to 2 ml ampules, frozen

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* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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8. Eisen, H. N., and Pressman, D., *J. Immunol.*, 1950, v64, 487.

9. Loeb, E. N., Knowlton, A. I., Stoerk, H. C., and Seegal, B. C., *J. Exper. Med.*, 1949, v89, 287.

in a dry ice-cello-solve solution and stored at –70°C. Before use, the serum complement was destroyed by heating one half hour at 56°C and the serum was absorbed 4 times with rat red blood cells and plasma. It was found that the injection intravenously of 2 ml into a 150 g rat would cause death through renal failure in about 2 weeks. This dose was given on one day or on 2 consecutive days. The onset of nephritis was almost always within 24 hours although infrequently it was delayed until 96 hours.

II. *Methods of absorption.* 2 ml amounts of serum were used in assaying the ability of various fractions of the kidney to absorb the nephrotoxic antibody. 0.4 ml of material to be tested for this capacity was added to the serum and allowed to stand in the refrigerator from 4 to 9 hours. The serum was then centrifuged at 2,500 r.p.m. for 15 minutes and the precipitate discarded. Four or five such absorptions were done, making the total amount of material added about 1.6 ml. Almost all kidney material tested gave precipitation, regardless of the extent of removal of nephrotoxic antibody. Hence it would appear that many antibodies were present which were not harmful to the rat. When solid material was assayed for antigenic activity, 4 absorptions were also done.

III. *Examination of rats.* Wistar rats of weight 150 g to 250 g were used. The absorbed sera were injected intravenously by employing the veins of the tail or feet. Nephritis was tested for by quantitative 24 hour urine protein determinations using the Shevky-Stafford method(10), and by histological examination of the kidneys. These two methods corroborated each other consistently. Twenty-four hour urine specimens were collected in a urine-feces separator and one drop of formalin was added as a preservative. A series of normal controls established that the 24-hour output of protein ranged from 0 to 6 mg. Since an occasional larger rat excreted as much as 10 mg, a 24-hour excretion of 10 mg of protein per 24 hours was taken as the upper limit of normal. In contrast to this finding, rats with nephritis usually excreted over 100 mg of protein daily. Animals were fasted during urine collections. The rats were killed by ether anesthesia. The kidneys were fixed in 10% formalin and were stained with hematoxylin and eosin and with the Hotchkiss connective tissue stain. The nephritis produced in our rats was essentially the same as that described by Smadel(2-3). Early histological changes consisted essentially of enlarged glomerular tufts with thickened basement membranes and few erythrocytes in the glomerular capillaries. There was a slight increase in the cellularity of the tufts.

Experimental. I. *Experiments with whole kidney.* Suspension of whole kidney readily absorbed out the nephrotoxic antibodies. A suspension of half of a kidney was found to absorb the antibodies present in 2 ml of serum. Lyophilized kidney was equally as effective. Lyophilized kidney was placed in a Soxhlet apparatus and extracted with a mixture of ether-alcohol, 1 part to 3 parts, for 8 hours. The material still retained its ability to absorb out the nephrotoxic antibody from serum. Because of the results described by Loeb *et al.*(9), the capacity of rat placenta to absorb sera was tested. Two ml of serum was absorbed with a total of 4 ground-up placentas which had been obtained from rats almost at parturition. The absorbed serum failed to produce nephritis. Nine rats were protected in this manner. In contrast to these findings, 2 placentas failed to absorb the nephrotoxic antibodies from 2 ml of serum. The antigen responsible for the nephrotoxic effect thus appears to be present in the placenta, though possibly at a lesser concentration than in kidney tissue.

II. *Experiments with solvents.* The extraction of kidney with water, saline, alkaline and acid solutions, alcohol or diethylene glycol failed to bring the antigen into solution. Animals subject to test with sera which had been absorbed with these materials invariably developed nephritis. An extract of an autolysate of kidney prepared by incubation at 37°C for 36 hours did not contain the antigen.

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10. Kolmer, J. A., and Boerner, F., *Approved Laboratory Technique,* Appleton-Century-Crofts, Inc., N. Y., Fourth Edition, 1945, p. 133.

TABLE I. Effect of Absorption of Nephrotoxic Serum with Specific Rat Kidney Soluble Substance upon Excretion of Urinary Protein.

	Rat No.	Source of material used for absorption	24 hr urinary protein output Days after injection				
			5	6	10	11	12
			mg	mg	mg	mg	mg
Test rats	1	Kidney		6	6	6	
	2			4		9	
	3			4	6	8	
	4			1	0	0	
	5			2		8	
	6			1	0	0	
Control rats	7	Muscle	147			Slight ascites	311
	8		61			Anasarca	151
	9		147				144
	10		116			”	151
	11		130				173
	12		130			Ascites	151

III. *Digestion of kidney with trypsin.* These experiments led to the production of the specific soluble substance in solution and hence will be described in detail. Twelve kidneys, totaling about 12 g in weight, were ground in a mortar, made into a thick paste with 0.85% sodium chloride and buffered with 0.1 M sodium borate to pH 8. To this was added 100 mg of crystalline trypsin. The suspension was placed in a 37°C incubator and stirred frequently. Digestion by the trypsin caused the pH to drop rapidly. Hence a pH determination was performed every half hour and borate added to restore the pH to 8. Incubation was continued for 3 hours, at the end of which time little drop in pH was being obtained. The suspension was then heated in a water bath at 60°C for 30 minutes to inactivate the trypsin. Since the volume of the suspension was about 30 ml another 10 ml of 0.85% sodium chloride were added so as to fill the large cup of a Spinco Preparative Model "L" ultracentrifuge. The suspension was centrifuged at 27,000 r.p.m. for 35 minutes. This made available a mucoid, homogeneous, clear tea-colored supernatant fluid which was pipetted from the precipitate. It was found that a total of 0.75 ml of this clear supernatant would precipitate with 2 ml of serum to remove the nephrotoxic antibodies. This supernatant in 5 portions, was added at intervals of from 4 to 8 hours. At the time this report was written a total of 24 rats had been protected from nephritis by precipitating antibodies from the sera with this material before injection. Five different batches of the material were prepared by the above method and each was found to be effective.

Three types of control experiments were carried out to exclude trypsin activity as the protective agent: (1) Twenty-five mg of trypsin were dissolved in 10 ml of rat plasma and a pH 8 was maintained by the addition of a small amount of 0.1 M sodium borate. Three samples of serum were absorbed with this solution and injected into rats, all of whom developed nephritis. (2) To 2 ml of serum was added 10 mg trypsin and the serum was then absorbed 4 times of 4 hours each with rat red blood cells. This serum was nephrotoxic to a rat. (3) In the last control experiment the procedure for obtaining specific soluble substance as described above was followed except that 12 g of muscle from the thigh and abdomen were substituted for 12 g of kidney. A preparation of kidney material was made at the same time. Six sera were absorbed with the supernatant from muscle at the same time that 6 sera were absorbed with supernatant from the kidney preparation. The twelve sera were injected into rats of 250 g on the same day. The results are given in Table I. It can be seen that the trypsin-digested kidney preparation gave uniform protection to 6 rats. On the other hand the trypsin-digested muscle preparation failed to protect; all 6 animals developed nephritis.

Some protected rats have been kept for periods of up to 3 weeks without developing proteinuria or kidney lesions by histological examination.

IV. *Further purification of the supernatant of trypsin-digested kidney.* The results of further attempts to isolate the specific soluble substance now in progress will be reported in a later communication. Suffice it to say at present that the substance is stable for several weeks at alkaline pH in the refrigerator, and that it is not sedimented at 35,000 r.p.m. for 120 minutes in the Spinco 40 preparative centrifuge.

Discussion. The studies described above suggest that the antigen involved in the production of nephrotoxic nephritis is a substance bound within the tissues of the kidney by protein molecules, and hence cannot be obtained in solution by simple extraction with solvents. Digestion with trypsin probably destroys the protein which binds the specific soluble substance herein described and permits it to escape into solution. Possibly the antigen concerned is a protein in combination with a polysaccharide or lipid and a portion of this complex molecule is resistant to trypsin because of the non-protein components. We are at present attempting to produce nephrotoxic antibody by injecting this soluble material into rabbits, and our preliminary results indicate that the material is not antigenic. If no such antibody is produced, we may assume that trypsin digestion splits off a hapten group from a larger antigenic molecule. If antibodies can be produced to this specific soluble substance the entire antigen molecule must exist in solution.

Nephrotoxic nephritis is an unusual experimental phenomenon in that one insult to the kidney can produce a chronic and progressive disease. Isolation of the specific antigen or hapten in purified form will permit more precise studies of this experimental nephritis. Certainly it must have some unique properties because only antibodies to it will produce this type of nephritis. Antibodies to the many other antigens in the rat kidney do not produce this picture. The nature of this specific substance should be of interest in the study of certain diseases such as rheumatic fever, glomerulonephritis, and periarteritis nodosa, which are thought likely to be on an allergic basis. If these human diseases are due to the action of an "autoantibody" upon tissue antigen or hapten, the antigenic substance may be of similar nature to that which produces nephrotoxic antibody when injected into rabbits. At least, it does not seem surprising that attempts in the past to find antibodies to saline extracts of the heart muscle in rheumatic fever patients and to saline extracts of kidney tissue in glomerulonephritis patients, have been fruitless.

Summary. A specific soluble substance which will combine with the nephrotoxic antibody of anti-rat kidney serum was obtained in solution. The material was liberated into solution by digesting rat kidney with trypsin at pH 8. This substance was assayed by its ability to absorb antibodies from nephrotoxic serum. It protected a total of 24 rats without failure. Control experiments excluded trypsin activity as being the protective substance. Further characterization should be possible now that this specific material has been obtained in solution.

Received June 15, 1951. P.S.E.B.M., 1951, v77.

Effect of Vitamins E, B_{12} and Folacin on CCL_4 Toxicity and Protein Utilization in Rats.* (18828)

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Popper, Koch-Weser, and Szento(1) reported that vit. B_{12} protected against carbon tetrachloride damage to livers in rats maintained on an unspecified diet. Previously Hove(2) had shown that vit. E protected rats against death due to CCl_4 when the animals were reared on a 10% casein diet deficient in vit. E. Since vit. B_{12} and folacin were not added to this diet, it became of interest to determine the influence of these vitamins on the protective action of vit. E against acute CCl_4 poisoning.

Experimental. The diets contained a 10% protein level as furnished either by water-washed commercial casein or by commercial solvent extracted soybean meal. The remainder of the diet consisted of salt mixture 4%, lard 9%, cod liver oil 1%, and sucrose to 100%. Pure vitamins were added in amounts such that each gram of diet contained: thiamine, riboflavin and pyridoxine, 5 μg each; calcium pantothenate, 25 μg; niacin 40 μg; choline chloride, 2 mg; i-inositol, 0.2 mg; and 2-methyl 1-4 naphthoquinone, 2 μg. These diets were supplemented as indicated with DL-α-tocopheryl acetate, vitamin B_{12} as furnished by a Fuller's earth concentrate containing 120 μg/g (Merck), and folacin as Folvite (Lederle).

Male rats of the Sprague-Dawley strain were placed on the diets and supplements, *ad libitum*, at weaning (40 to 50 g) and maintained for periods of 4 to 12 weeks in individual cages. Weekly body weight records were kept, and food consumption was measured for the rats on the soybean meal diets. Protein efficiency ratios were calculated for 4-week periods by dividing the total gain in body weight by the total protein ingested. Presumably this represents the efficiency with which the animals utilize dietary protein for growth.

The sensitivity of the rats to CCl_4 was determined by injecting this substance intraperitoneally at the levels indicated, and noting the times at which deaths occurred. Observations were made for 1 week but, as noted previously(2), most deaths came within 48 hours and therefore the data have been expressed as the number and per cent survivals at this time. The single CCl_4 injections were made after the rats had been on the various diets for periods ranging from 4 to 12 weeks. The age of the animals at the time of injection appeared not to influence the results and this factor has been ignored in combining the data. As determined in preliminary studies, both the level and duration of the tocopherol supplementation had a distinct bearing on the results. It was noted that the 0.01% dietary tocopherol level had to be fed for at least 2 weeks prior to poisoning to obtain protection. However, a single dose of tocopherol, if high enough, afforded protection; 25 mg given orally to 12 rats 2 days prior to the CCl_4 permitted survival of 8 (67%), but 5 mg so administered was without benefit. Therefore, all supplemented diets were fed for at least 4 weeks in the presently reported experiments.

Results. Protein utilization of rats on the soybean meal basal diet was significantly improved by the α-tocopheryl acetate supplement (Table I). A similar effect, noted with the casein diet (Table II), was reported pre-

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* Published with the approval of the Director of the Alabama Agricultural Experiment Station. This work was performed under Contract No. N9 onr92100 with the Office of Naval Research. Part of the results were presented at the annual meeting of the Fed. Am. Soc. for Exp. Biol., 1950, *Fed. Proc.*, v9, 362. Folacin was donated by Lederle Laboratories and the other vitamins by Merck and Co.

1. Popper, H., Koch-Weser, D., and Szento, P. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 688.

2. Hove, E. L., *Arch. Biochem.*, 1948, v17, 467.

TABLE I. Protein Utilization and CCl_4 Sensitivity of Rats on 10% Soybean Protein Diet.

Additions to 100 g of soybean diet	No. of rats	Wt gain 4 wk, g	Protein efficiency ratios		Survivals 48 hr after levels of CCl_4/kg, of: 2 ml	1.5 ml	1 ml
					%	%	%
None	33	26	1.75	.064*	2/20 (10)	3/7 (43)	3/6 (50)
α-tocopheryl acetate, .01 g	23	34	2.29	.046	15/18 (83)	—	5/5 (100)
Vitamin B_{12}, 3 μg	16	31	2.03	.081	1/9 (11)	—	4/7 (57)
Vit. B_{12} and toc. acet.	11	27	1.88	.073	6/6 (100)	—	5/5 (100)
Folacin, 1 mg	7	18	1.62	.105	—	1/7 (14)	—
Folacin and vit. B_{12}	11	37	2.47	.062	0/5 (0)	2/6 (33)	—
Folacin, vit. B_{12} and tocopheryl acet.	11	38	2.59	.076	3/5 (60)	6/6 (100)	—

* Std error of mean.

TABLE II. Growth and CCl_4 Sensitivity of Rats on a 10% Casein Diet.

Additions to 100 g of casein diet	No. of rats	Wt gain 4 wk, g	Survivals 48 hr after levels of CCl_4/kg, as follows: 2 ml	1.5 ml	1 ml
			%	%	%
None	72	34	6/28 (21)	4/17 (24)	13/27 (48)
α-tocopheryl acetate, .01 g	70	40	24/29 (83)	12/14 (85)	25/27 (93)
Vit. B_{12}, 3 μg	21	44	12/16 (75)	—	4/5 (80)
” and toc. acet.	20	53	13/15 (87)	—	5/5 (100)
Folacin, 1 mg	10	26	—	2/10 (20)	—
” and toc. acet.	10	48	—	10/10 (100)	—
Aminopterin, 70 μg	14	25	—	2/14 (14)	—
” and toc. acet.	19	37	—	16/19 (85)	—
L-cystine, .1 g	11	52	0/5 (0)	—	6/6 (100)
” .3 g	10	51	—	—	9/10 (90)
” 1 g	16	68	7/10 (70)	—	5/6 (85)
Cystine, .3, and toc. acet.	8	74	—	—	7/9 (78)
DL-methionine, .1 g	6	56	—	—	5/6 (83)
Methionine and toc. acet.	6	62	—	—	5/6 (83)
Theophylline, .1 g	4	33	—	—	4/4 (100)
” and toc. acet.	4	42	—	—	4/4 (100)
Creatine, .5 g	8	39	0/8 (0)	—	—
” and toc. acet.	8	53	7/8 (88)	—	—
Guanidoacetic acid, .5 g	8	29	4/8 (50)	—	—
” and toc. acet.	8	31	2/8 (25)	—	—

viously(3-4). Supplements of vit. B_{12} improved protein utilization on the vitamin E-low soybean diet, but folacin supplements were ineffective. On this diet the combined supplement of vit. B_{12} and folacin resulted in a protein efficiency value that was significantly higher than that given by vit. B_{12} alone. The addition of tocopherol together with the vit. B_{12} and folacin resulted in a still higher value.

Excellent protection against the acute CCl_4 toxicity in the rats was conferred by supplementation of either diet with vit. E (Tables I and II). Dietary vit. B_{12} protected rats on the casein basal diet against the lethal effect of CCl_4, but on the soybean meal basal diet this vitamin, at the level fed, was completely ineffective, even when combined with folacin. Folacin supplements alone were without benefit to rats on either diet. The major treatment effects (Table I) may be summarized as follows: On the soybean meal basal diet, the supplements of vit. B_{12}, folacin, or both, allowed survival of 8 out of 34 rats (23%). On the same diets to which tocopherol was added, 40 out of 45 rats (89%) survived. Similarly, on the casein basal diet (Table II) supplemented with

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3. Hove, E. L., and Harris, P. L., *J. Nutrition*, 1947, v34, 571.

4. Moore, T., *Annals N. Y. Acad. Science*, 1949, v52, 206.

tocopherol, 113 out of the 129 rats (88%) survived, and on the casein diets to which vitamin B_{12} was added, 16 of the 21 rats (76%) lived past the 48-hour point.

These data indicate that the protective effect of the vit. B_{12} against acute CCl_4 toxicity depends upon the nature of the basal diet, since it was positive on the casein diet but negative on the soybean diet, at the levels fed. However, an explanation of this discrepancy may be found on considering the dosage levels. A dietary vit. B_{12} level of 3 μg/100 g diet has been considered fully adequate for the rat, and indeed this level did significantly improve the protein utilization in rats on the soybean diet (Table I). The requirement of vit. B_{12} for protection against CCl_4 may be higher than the level used. To test this, an experiment was set up in which vit. B_{12} was added at the levels of 0, 3, and 30 μg/100 g of each basal diet. After injecting CCl_4 (2 ml/kg body weight) into rats reared on these diets, it became evident that the higher (30 μg) level of vit. B_{12} protected rats on the soybean diet, but that the "normal" (3 μg) level did not; the values for survival were 6 out of 8 rats (75%) and 1 out of 5 rats (20%), respectively. Survival in the control group was also 1 out of 5. Survival of rats on the casein diets were 80% on the 3 μg level, and 66% of the rats on the 30 μg level of vit. B_{12}. Growth of the rats was not increased by the higher level of this vitamin as compared with the lower level.

It was previously noted(2) that methionine and theophylline added at a 0.1% level to the casein diet deficient in vit. E gave excellent protection against CCl_4. This is confirmed by data in Table II. Dietary creatine was without benefit, while dietary guanidoacetic acid appeared from limited data to suppress both growth and the protective action of vit. E.

L-Cystine gave fairly good protection against acute CCl_4 toxicity (Table II). Preliminary results showed that the partial protection given by the lower levels of cystine and tocopherol was not improved by combining these supplements (Fig. 1). Experiments are currently underway to determine the possible relation of vit. B_{12} and folacin to the protective effect of the sulfur amino acids.

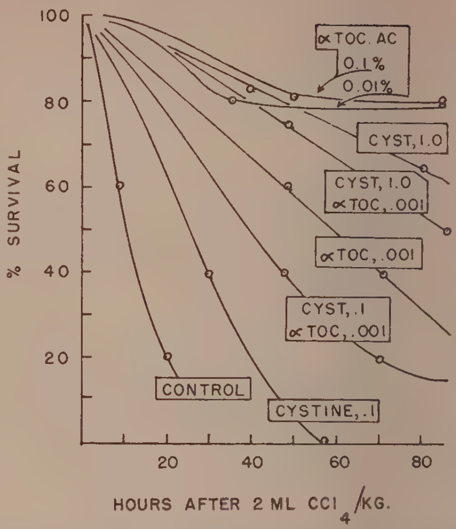

FIG. 1. Influence of various percentage levels of cystine and DL α-tocopheryl acetate added to the 10% casein diet, on susceptibility of rats to acute CCl_4 toxicity. Five to 6 male rats per group on the diets and supplements for 6 weeks from weaning.

Discussion. An interrelation of vit. E and vit. B_{12} has been noted in that they were mutually replaceable when using as criteria either the increased protein utilization or the protection of rats on 10% protein diets against the lethal effects of CCl_4. Apparently the rat has a much higher vit. B_{12} requirement for protection against CCl_4 than it does for growth. On the soybean basal diet a good protein utilization response was obtained with vit. B_{12} added at a level of 3 μg/100 g, but this had to be increased to 30 μg/100 g to get protection against CCl_4. Folacin supplements alone did not have an influence on protein utilization, but the data suggest that this vitamin influences the results obtained with vit. B_{12} and must be added to get maximum effect, as has been noted previously by Schaefer and co-workers(5) in work relating these vitamins to transmethylation and the choline requirement.

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5. Schaefer, A. E., Salmon, W. D., Strength, D. R., and Copeland, D. H., *J. Nutrition,* 1950, v40, 95.

Presumably, the casein basal diet contained enough vit. B_{12} as an impurity in the casein so that the additional supplement of 3 μg/100 g was sufficient for protection against the CCl_4. Growth of rats on the casein basal diet was not significantly improved by supplementation with vit. B_{12} alone. The apparent growth improvement seen in the data in Table II was not nearly so evident when comparison was made with controls from the specific experiment. Dilution of these control values with large numbers of control values from other experiments was done to emphasize the lethal influence of CCl_4 since this was not affected by growth or body size of the rats.

Goyco(6) recently reported on the beneficial effect of vit. B_{12}, vit. E and methionine on utilization of yeast protein by rats. His results were similar in many respects to the data obtained with the soybean meal protein diet and herein reported.

Summary. Vit. E and vit. B_{12} can replace each other in promoting protein utilization and in protecting against acute carbon tetrachloride toxicity in young rats reared on 10% protein diets deficient in these factors. The vit. B_{12} requirement for protection against CCl_4 is considerably higher than for growth. Folacin supplements, alone, had no effect, but, in the presence of vit. B_{12} or vit. E, this factor markedly improved protein utilization and growth. L-Cystine protected against the acute toxicity but a synergism between cystine and vit. E was not noted.

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Received June 18, 1951. P.S.E.B.M., 1951, v77.

Inhibition of Influenza and Mumps Viruses in Tissue Culture by Basic Amino Acids.* (18829)

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Recent studies(1-4) have indicated that modification of the host-cell metabolism by a variety of substances may interfere with the growth of animal viruses in tissue culture. Emphasis has been placed on inhibition of viruses by metabolic antagonists such as methoxinine and malonic acid(1-3) but an excess of amino acids may also inhibit virus multiplication as exemplified by the effects of lysine and tryptophane on a neurotropic virus in mouse brain tissue cultures(1). In the present study inhibition of the growth of influenza and mumps viruses by arginine and other amino acids was observed. Investigations on the effects of these substances on the viruses and tissues themselves are reported.

Methods. Tissue cultures of minced chorioallantoic membrane of 10 to 12 day old chick embryos in balanced salt solution with glucose and sodium bicarbonate were prepared according to methods previously described(4-5). Allantoic passage strains of mumps and influenza (PR8 and Lee) were inoculated at dilutions of 10^{-2} into tissue cultures containing the substances to be tested. Cultures containing mumps and the PR8 strain of influenza were incubated at 35°C, those with the Lee strain at 31°C. Virus multiplication was measured by hemagglutination titrations with chicken erythrocytes

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* This work was aided by grants from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, the Eugene Higgins Trust, and the William F. Milton Fund.

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2. Ackermann, W. W., *J. Biol. Chem.*, 1951, v189, 421.

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4. Eaton, M. D., Cheever, F. S., and Levenson, C. G., *J. Immunol.*, 1951, v66, 463.

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TABLE I. Effect of Amino Acids on Growth of Influenza B in Tissue Cultures.

Substance	Conc., mg/ml	6 day hemagglutination titers		Ratio control/test
		Test	Control	
l-arginine	5	12	60	5
	5*	2	100	50
	2	15	39	2.6
dl-lysine	5	3	65	22
	2	6	45	7.6
	2*	2	70	35
dl-ornithine	10	<1	100	>100
	2	80	100	1.3
Guanidoacetate	10	12	100	8.3
	2	32	100	3.1
dl-histidine	5	38	77	2
Creatine	5	56	34	.6
Protamine	1	16	45	3

* Buffered at pH 7.8-8.0. In remaining experiments a drop in pH to 7.0 or 6.8 occurred over a period of 6 days.

and in some instances by infectivity titrations.

Results. The results of experiments with the Lee strain of influenza B are summarized in Table I. L-arginine and dl-lysine have been studied most extensively and were found to produce some inhibition at concentrations down to 1 or 2 mg/ml. Inhibition of formation of viral hemagglutinins was marked with ornithine and moderate with guanidoacetate at 10 mg/ml. Histidine produced only slight effects while results with creatine at the concentrations tested were negative. Small effects similar to those with histidine were seen with other amino acids such as glycine, and also with asparagine. Protamine at the maximum non-toxic concentration of 1 mg/ml did not markedly effect the growth of virus. L-lysine was found to be approximately twice as active as the dl-form. Lysine and arginine at concentrations of 10 mg/ml inhibited the growth of influenza A when this was inoculated at dilutions of 10^{-3} but had little effect against larger inocula. With influenza B addition of 5 mg/ml of arginine 24 hours after the virus produced the same degree of inhibition as when added immediately, but no effect was observed when the period was increased to 48 hours. Infectivity titrations in representative experiments indicated that arginine and lysine reduced the infective property of influenza B in tissue cultures to about the same extent as hemagglutination.

Inhibition of the Lee strain by lysine and arginine was favored by using cotton stoppered flasks and adding enough bicarbonate buffer to the medium to maintain the pH between 7.8 and 8.0 (Table I), also by omission of phosphate from the medium. No reversal of the effect of arginine was obtained with ribose nucleic acid, adenylic acid, methionine or creatine, but preliminary experiments suggest that phospholipids may have some reversing activity since virus grows normally in cultures containing 5 mg/ml of arginine and 10% of egg yolk.

Extension of these observations to mumps virus which grows more slowly gave a better insight into the kinetics as will be seen in Fig. 1. Although a single addition of 10 mg of arginine at the beginning of the experiment was without effect, changing the fluids so as to add fresh amounts of arginine at 4 days resulted in inhibition as did delay in the addition of arginine until the 4th day after inoculation. In the cultures containing arginine some growth of mumps virus was observed between the 12th and 20th day and removal of the arginine by changing the fluids on the 8th day to balanced salt solution without arginine resulted in more active growth of virus by the 16th day. The loss of effect with time does not seem to be due to disappearance of the arginine because no marked diminution could be detected by assays with an arginine requiring mutant of *E. coli*.

No direct inactivating effect of arginine on influenza virus *in vitro* could be demonstrated. When dilutions of virus in balanced salt solution were incubated at 31°C with arginine at a concentration of 20 mg/ml infectivity disappeared less rapidly than in controls without arginine. Similar concentrations of arginine when added to dilutions of mumps or influenza virus plus chicken erythrocytes did not alter the hemagglutination end point. Arginine at concentrations of 5 mg/ml did not prevent the adsorption of virus to chorioallantoic membrane either at 37°C or at 4°C.[†]

Exposure of fragments of chorioallantoic membrane for periods up to 16 days to ar-

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[†] We are indebted to Mr. Robert Gohd for these determinations.

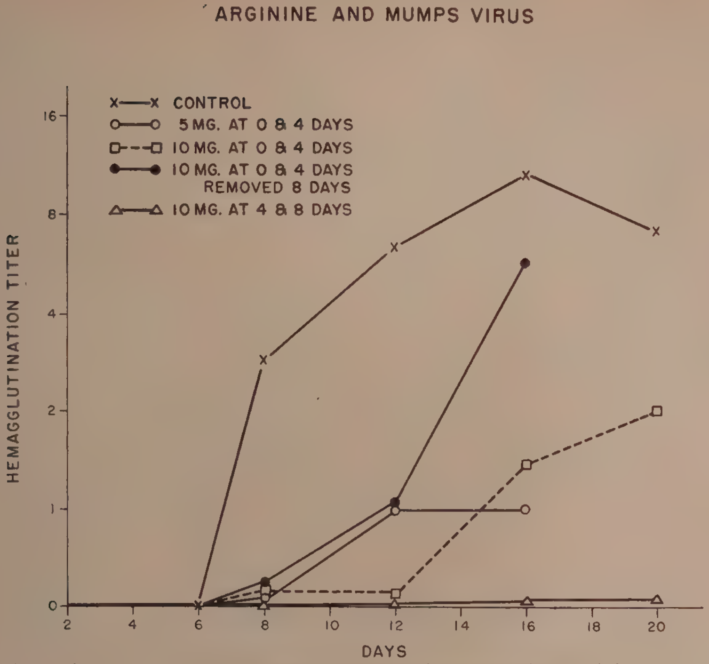

FIG. 1. Open circles and squares; balanced salt solution containing arginine changed to same solution at 4 days. Solid circles; same but additional change to balanced salt solution without arginine at 8 days. Triangles; started with balanced salt solution only and changed twice to arginine 10 mg/ml at 4 and 8 days. Control curve represents avg titers of series with and without change of solution.

ginine at concentrations of 10 mg/ml or lysine at 5 mg/ml did not reduce the ability of the cells to grow after explant to a fibrin clot medium in roller tube cultures. In such fragments the outgrowth of epithelial tissue and fibroblasts occurred in the same manner as in the controls. Concentrations of arginine and lysine of 20 mg/ml were lethal to tissue. When the amino acids were added directly to the roller tube cultures retarded growth of tissue was observed with lysine at 10 mg/ml but was less evident with arginine.

Since some inhibitors of virus growth were shown to reduce tissue respiration(2) the effect of arginine on oxygen uptake of the chorioallantoic membrane suspended in buffered salt solution was determined in the conventional Warburg apparatus. Respiration was found to proceed at the same rate in the presence of arginine at a concentration of 5 mg/ml as in controls without arginine. Exposure to arginine in virustatic concentrations for as long as 6 days did not affect the rate of oxygen uptake. Similarly the rate of disappearance of glucose in tissue cultures measured over a period of 6 days was identical in the presence and absence of arginine. In contrast to some other inhibitors, arginine does not seem to exert its virustatic action by affecting the respiratory activities of the host cell.

In preliminary experiments no effect of arginine in virustatic concentrations on the growth of *A. aerogenes* in a minimal medium containing glucose could be found, and the capacity of this organism to adapt to growth on mesoinositol was not affected(6).

Discussion. Although several different amino acids will reduce the rate of viral multiplication in tissue cultures those containing 2 amino groups appear to be the most active. Arginine which has been studied in most detail

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probably inhibits the growth of influenza and mumps viruses by some effect on the host cell or on the interaction of host cell and virus. This effect is reversible by removing the amino acid and is brought about in the absence of demonstrable changes in the oxygen consumption, glucose utilization, or growth potential of the tissue. The reversal of the effect of arginine by egg yolk suggests the possibility of interaction with lipids in the cell membrane. The augmenting effect of an alkaline reaction (pH 8.0) is similar to that seen with basic antibacterial substances such as streptomycin and diamidines(7). Synthetic lysine polypeptides have been reported to inhibit the growth of influenza virus(8), but these cause aggregation of virus particles, bacteria and red cells while arginine and lysine apparently have no direct effect either on the infective or hemagglutinating properties of mumps and influenza viruses.

Summary. Arginine, lysine, ornithine, and to a lesser extent some other amino acids retard the growth of influenza and mumps viruses in tissue cultures when added at concentrations of 1 to 10 mg/ml. The virustatic effect of arginine and lysine could not be attributed to toxicity or to direct action of these amino acids on the viruses.

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Received June 22, 1951. P.S.E.B.M., 1951, v77.

Failure of an Ergot Preparation to Shorten Coagulation Time in Hemophilia.* (18830)

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Vodopivec(1) describes a striking acceleration of the clotting of the blood of hemophilics by administration of a mixture of equal parts of 3 hydrogenated ergot alkaloids, namely dihydroergocristine, dihydroergocornine, and dihydroergokryptine. This preparation, known also as Hydergine or CCK-179, was reported to be effective orally in dosage of 1 mg every 3 hours and parenterally after a single injection of 0.6 mg. The clotting time fell to normal 2 to 3 hours after oral, 1 to 2 hours after subcutaneous, and 30 minutes after intravenous administration. The effect persisted for a day after the drug was given orally, and for several hours after parenteral treatment. Although we are ignorant of other observations on the use of ergot derivatives in hemophilia, several papers(2-4) discuss the adrenergic blockade effected by Hydergine and its several components.

Because great clinical interest attaches to any pharmacologic agent able to accelerate coagulation in hemophilia, we have examined the effects of Hydergine[†] in 4 hemophilics.

Methods. Coagulation time in glass at 37.5°C was determined by the method of Pohle and Taylor(5). One-stage prothrombin levels were measured as described by Rosenfield and Tuft(6), and 2-stage determinations by the method of Ware and See-

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[*] This investigation was aided by a grant from the Blood Grouping Laboratory of Boston, Inc.

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gers(7). Serum prothrombin was measured on serum separated one hour after clotting and incubated at 37.5°C for 24 hours, using barium sulfate plasma as diluent. Labile factor was determined in plasma by the method of Stefanini(8). The recalcification time of oxalated plasma was performed according to Quick(9), with the modification of adjusting the pH to 7.35 by equilibrating the sample with a gas mixture of 5% carbon dioxide and 95% oxygen before recalcifying. Fibrinolytic activity was tested by observation of fibrin clots formed by addition of 0.2 ml each of 0.02 M $CaCl_2$, Difco thromboplastin, and of oxalated plasma to 5 ml of imidazole-buffered saline (pH 7.25). Lysis within 24 hours at 37.5°C was considered abnormal. Proteolytic activity was also measured after the method of Tagnon(10), using bovine fibrinogen tagged with radio-active iodine as substrate.

The 4 hemophilics were well established as to diagnosis and had been frequently treated at this hospital for the usual sequels of the disease. They were normotensive, and reduction of the coagulation time of their blood promptly followed the addition of normal blood *in vitro* or *in vivo*. One patient, Case 1, has frequently been hospitalized by hemophilic arthropathy, while the others, Cases 2, 3, and 4, were admitted to the metabolic ward for the purpose of this study. Before Hydergine administration one or more base-line determinations of pulse and blood pressure were made and the coagulation tests were performed. Case 1 received Hydergine 1 mg orally every 4 hours for 3 days, followed by 1 mg orally every 3 hours for 1 day, and finally 0.5 mg intravenously. Cases 2 and 3 each received 0.6 mg intravenously once. Case 4 was given 1.2 mg intravenously once. The coagulation tests were performed daily during the period of oral treatment. Following the intravenous administration they were repeated at approximately hourly intervals for 3 hours, and again at 24 hours.

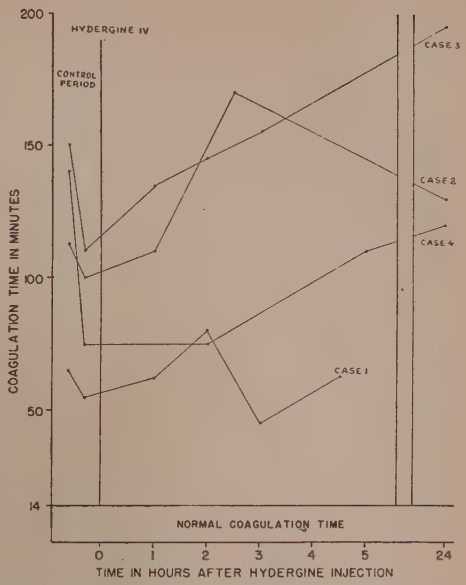

FIG. 1. Coagulation time in 4 hemophilics following intravenous administration of Hydergine. Case 1 received 0.5 mg; cases 2 and 3 received 0.6 mg; case 4 received 1.2 mg.

Results. In Case 1 during oral treatment a decline in clotting time from 69 to 40 minutes occurred. However, this was probably insignificant as there was no shortening of clotting time in any of the 4 patients following intravenous Hydergine (Fig. 1). Of the 3 patients receiving 0.5 or 0.6 mg intravenously, the recalcification times (Fig. 2) decreased slightly in 2 at the first hour after injection, while there was no change in the third patient. In Case 4, who received 1.2 mg intravenously, there was a more marked decrease in the recalcification time, which was found to be 135 seconds 2 hours after administration of the drug. Thereafter the value fluctuated from 270 seconds at 5 hours to 135 seconds at 24 hours. The one-stage prothrombin level was not significantly altered in any patient after intravenous Hydergine; the two-stage prothrombin content declined slightly at the second hour after injection in the 2 patients in

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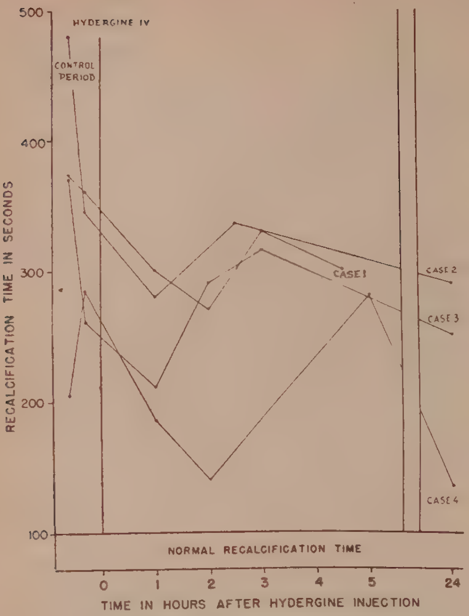

FIG. 2. Recalcification time in 4 hemophilics following intravenous administration of Hydergine. Case 1 received 0.5 mg; cases 2 and 3 received 0.6 mg; case 4 received 1.2 mg.

whom it was determined. Of the 3 patients receiving the intravenous dose of 0.5 or 0.6 mg the serum prothrombin decreased moderately in 2, though never to levels below 50%, while it was unchanged in the third. In the fourth patient, who received 1.2 mg intravenously, it changed from an original value of 60% to a final value of 20%. The decline was gradual and did not correlate with the variations in recalcification time. Lysis of the diluted plasma clot occurred in a single specimen taken after 24 hours of oral treatment in Case 1 and in specimens from Case 4 at 2 and at 5 hours after the 1.2 mg injection. In the patients receiving 0.6 mg intravenously, none of numerous clots dissolved. Using fibrinogen tagged with radioactive iodine as a substrate, samples of plasma from Cases 2, 3, and 4 showed no increase in lysis after Hydergine administration. Plasma labile factor was insignificantly altered in any patient in the first 5 hours after injection. Only the patient who was given 1.2 mg of Hydergine intravenously experienced unpleasant symptoms. These consisted of nausea and vomiting shortly after injection, severe nasal congestion, and frontal headache which lasted about 6 hours. Alterations in pulse and blood pressure were not remarkable in any patient.

Summary and conclusions. Three hemophilics were treated with Hydergine according to a regimen reported to effect a marked reduction of clotting time. A fourth patient received twice the amount of drug stated by Vodopivec to be effective upon intravenous administration. No important acceleration of coagulation was noted. Only in the patient who received an excessive dose and became ill were there any impressive changes. These consisted of a transient decrease in recalcification time and a gradual decline in serum prothrombin, as well as fibrinolysis *in vitro*. Since the Hydergine used in this study is believed to be identical to that administered by Vodopivec, we are unable to explain our inability to confirm his findings.

The authors are indebted to Miss Helen Corrigan and Mrs. William Stephan for technical assistance.

Received June 22, 1951. P.S.E.B.M., 1951, v77.

Effect of Starvation on Phagocytosis *in vivo*.[*] (18831)

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Phagocytosis has long been known to bear a relationship to the nutritional status of the experimental animal. Many investigators have concluded that phagocytosis is depressed by the omission of some element of the food supply(1-4). On the other hand, others have shown that there is an increase in this activity when the food supply is decreased(5-8). In all of these reports, the animals had been depleted by long deprivation of one or more essential elements of the diet. Recent work, by one of the authors(9), carried out in tissue cultures, showed marked differences in motility, adhesiveness and preference for saccharides, between the leucocytes of starved and fed rats. These results suggested a study of the *in vivo* effect of starvation and phagocytosis.

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[*] Sponsored by Veterans Administration and published with the approval of Chief, Medical Director. Statements and conclusions published by the authors are a result of their own study and do not necessarily reflect opinion or policy of Veterans Administration.

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Methods. The phagocytic activity of rats deprived of food for 36 hours was compared with that of well fed animals. Both groups were injected parenterally with a suspension of bacteria and after intervals varying from 2-3¾ hours, peritoneal samples were removed and examined for phagocytic activity. The phagocytic activity was measured in terms of the percentage of W.B.C.-containing bacteria as well as the number of bacteria engulfed by the W.B.C. This work was conducted on mature male albino rats of the Wistar strain, weighing 275-375 g each. The phagocytic response was studied by using a non-hemolytic, coagulase positive strain of *Staphylococcus aureus* obtained from human isolation. The bacteria were grown on brain-heart infusion blood agar plates for a period of 18 hours at 37°C, at which time they were harvested and washed 3 times with saline. After partial dilution they were filtered through a thin layer of sterile absorbent cotton and then further diluted with saline to give approximately 50% transmission with a 400 mμ filter on the Coleman Junior Spectrophotometer. This suspension was stored in the refrigerator and just before use the next day, was warmed to body temperature and well shaken.

Inoculation of animals. The rats deprived of food for a period of 36 hours before inoculation are referred to as the starved group. Prior to starvation all the rats had received, for a period of 4 weeks, a complete diet in the nature of a plentiful supply of Rockland Farm Rat Pellets, supplemented by greens. Water was given *ad lib.* In general, experiments were performed on 5 starved and 5 fed animals simultaneously. Each rat was injected intraperitoneally with 20 cc of the prepared bacterial suspension. (None of the rats died as a result of the inoculation with the bacteria). After a minimal interval of 2 hours 3 to 8 cc of the peritoneal fluid were removed by alternately injecting air and then aspirating. A 10 ml syringe and a 19 gauge needle were used. The collected fluid was placed in a test tube and duplicate white cell counts were immediately made. At this time a sterile platinum loop of 5 mm diameter was dipped into the fluid and a 2 cm square smear made on one end of a glass slide. Two loops full were smeared over an equal area on the opposite end. Three such slides were made from each peritoneal specimen. The smears were allowed to dry and then were stained by a modified Gram stain. The crystal-violet was applied for not more than 15 seconds; the iodine for 10 seconds; the acetone-alcohol for 10-15 seconds, and the saffarin for 10 seconds.

Counting white blood cells and bacteria. Each of the duplicate W.B.C. counts were made in the usual way with a white-cell pipette and diluting fluid. Nine sq. mm were counted on each specimen and the average of the two replicates taken. The stained smears were counted in the following manner: Areas

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TABLE I. Comparison of the Phagocytic Activity of Starved and Fed Rats.

	No. of rats	WBC/cmm of perit. fluid ×100	% of Phago./100 WBC (stained smear)	Avg No. of bact. in Phago./100 WBC (stained smear)	Avg No. of Phago./cmm	Avg No. of bact. in Phago./cmm
Starved	21	14900	38	423	4563	52855
Fed	29	13700	19	129	2205	15344

TABLE II. Comparison of Phagocytic Activity of Groups of Rats Based on Time Interval Between Injection of *Staphylococcus aureus* and Withdrawal of the Peritoneal Sample.

Group	No. of rats	WBC/cmm ×100	% of phago./100 WBC	Avg No. bact. in phago./100 WBC	Avg No. phago./cmm	Avg No. bact. in phago./cmm
2 to 2½ hr group						
Starved	7	7.3	37	475	2839	31846
Fed	18	10.3	19	128	1488	10040
2¾ to 3¾ hr group						
Starved	14	17.5	38	399	5425	63358
Fed	11	19.4	20	131	3384	24028
Total of all animals						
Starved	21	14.9	38	423	4563	52855
Fed	29	13.7	19	129	2205	15344

were selected in which the extra as well as the intracellular bacteria were clearly distinguishable. Three hundred or four hundred consecutive W.B.C. were counted on each peritoneal sample and the number containing staphylococci as well as the number of bacteria in each cell was tabulated. The average number of phagocytosing W.B.C. was found by multiplying the percentage of phagocytosing cells by the total number of W.B.C. per cmm of peritoneal fluid. The number of bacteria engulfed was determined by multiplying the number of bacteria phagocytosed per 100 W.B.C. by the total (in hundreds) of W.B.C. per cmm.

Results. The data on the 21 starved rats and on the 29 fed animals is given in Table I. The significant features brought out by these data are the following: 1. The average number of W.B.C. per cmm of peritoneal fluid was essentially the same in both the starved and the fed groups, *i.e.*, 14900 and 13700. 2. The percentage of W.B.C. showing phagocytosis in the starved animals was twice that in the fed rats, *i.e.*, 38% and 19%. 3. The number of bacteria engulfed by the phagocytes of the starved rats per 100 W.B.C. was 3 times that of the fed group, *i.e.*, 423 and 129. Since all the rats were not aspirated exactly 2 hours after inoculation with *Staph. aureus,* it was thought that perhaps the length of time in the peritoneal cavity had some bearing on the results. Consequently, the rats were divided into two groups with regard to the time that elapsed between injection and aspiration. It may be observed (Table II) that although the 2¾-3¾ hour group had approximately twice as many W.B.C. per cmm as the 2-2½ hour animals, nevertheless, the starved groups showed twice the percentage of phagocytes, which engulfed three times the number of bacteria. That this relationship held despite the difference in time of aspiration and W.B.C. count, may be explained by assuming that at the time of the earliest aspiration, (2 hours) the peak outpouring of W.B.C. into the peritoneal cavity had been reached and that phagocytosis too was at the maximum. The subsequent rise in W.B.C. count was due to absorption of the injected liquid with consequent concentration of the cells present.

TABLE III. Distribution of Starved and Fed Rats in Relation to % of Phagocytosing WBC.

% of phagocytosing WBC	No. of starved rats	No. of fed rats
5–14	0	12
15–24	1	10
25–34	7	6
35–44	9	1
45–54	3	0
55–64	1	0
Total	21	29

TABLE IV. Distribution of Starved and Fed Rats in Relation to Avg Number of Bacteria Phagocytosed per 100 WBC.

Avg No. bacteria phagocytosed/100 WBC	No. of starved rats	No. of fed rats
24–99	0	13
100–199	2	12
200–299	4	3
300–399	5	1
400–499	2	0
500–599	3	0
600–699	3	0
700–799	2	0
Total	21	29

A comparison of the distribution of fed and starved rats on the basis of the percentage of phagocytosing W.B.C. is shown in Table III. It may be observed that 75% of the fed animals (22 of 29) fall into the lower range and 95% of the starved group (20 of 21) are found in the higher categories.

Table IV illustrates the difference in distribution between the 2 groups of rats on the basis of the average number of bacteria engulfed per 100 W.B.C. Here again the starved group far outdistanced the fed animals with 90% (19 of 21) rats showing a greater number of bacteria engulfed than 86% (25 of 29) in the fed group.

Discussion. The technics used in this report to determine *in vivo* phagocytic activity are an application of the *in vitro* methods of Wright(10), and Hamburger(10). Wright determined the number of particles engulfed by the leucocyte in a given time. The criterion used by Hamburger was the percentage of leucocytes showing phagocytosis in a definite period. In our experiments both the number of bacteria engulfed per phagocyte per unit of time and the percentage of leucocytes showing phagocytosis was determined. As the starved rats produced twice the percentage of phagocytic leucocytes which engulfed 3 times more bacteria than the phagocytes of fed animals, it can be seen that the results obtained with these *in vivo* technics are in agreement with each other and of a similar order of magnitude.

Inasmuch as there is difference of opinion with regard to the effect of starvation on phagocytic activity(1-3,9,11,12) it is not surprising that the explanations of this phenomenon are many and varied. It has been suggested that changes in temperature(13), vit. C (11,12), pH(14), complement(15), to mention just a few, were the cause of the rise or fall in phagocytic activity. More recently, Kuna and Chambers(9), as a result of their work in tissue cultures, observed marked differences in behavior between the leucocytes of rats starved for 36 hours and those from fed animals. The leucocytes of starved animals were attracted by mono, di and polysaccharides, whereas those of fed rats reacted only to polysaccharides. Starved rats showed a greater number of W.B.C. in motion as well as an increase in the rate of motion. The W.B.C. of starved rats were characterized by a greater degree of adhesiveness to particles in the media. The details of the mechanism whereby starvation induces greater phagocytic activity in rats, remains to be ascertained.

A number of observations are to be found in the literature, which emphasize the enhanced resistance to infection induced by dietary deficiency.

Rats on a B complex deficient diet survived infection with the blood flagellate (*Trypano-*

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some equiperdum) somewhat longer than control animals(6). Ascorbic acid deficiencies in monkeys markedly prolonged the course of an otherwise rapidly fatal infection with *Plasmodium knowlesi*(7). Healthy well nourished fowl were found to be more susceptible to virus chicken sarcoma than thin and ill ones(16). In experimental Murine typhus infection of guinea pig, the starved animals showed considerably less fever and scrotal reaction(8). Undernourished rabbits were less reactive to vaccine virus(17), and vitamin deficient mice failed to show characteristic signs of infection with Western equine encephalitis(18). In children, the original observation of Heine that poliomyelitis seems to attack the better fed children with relatively higher incidence has been confirmed(18).

In the above cited cases it would seem that lack of nutrition has rendered the host a relatively unsuitable culture medium for the parasite. Perhaps the increased phagocytic ability of the starved animals, as demonstrated by the rats in the experiment here reported, is a factor in rendering the host more resistant to invasion by the parasite.

Conclusions. Leucocytes obtained from rats well fed for 4 weeks prior to a 36-hour period of starvation were compared with leucocytes from continuously well fed rats. The starved group showed twice the percentage of phagocytosing W.B.C. which had engulfed 3 times the number of bacteria.

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Received May 23, 1951. P.S.E.B.M., 1951, v77.

A Rapid Method for Clinical Total Blood Volume Determination Using Radioactive Iodinated Human Serum Albumin (RIHSA)[*] (18832)

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This paper reports a rapid method of directly measuring blood volumes using radioactive iodinated human serum albumin as a tracer agent. Human serum albumin is iodinated with I_{131} (RIHSA) in an essentially neutral buffer. Each cc of RIHSA[†] contains 5 mg of human serum albumin and 2 mg of salt(4). Metabolic studies of RIHSA have been carefully done and reported elsewhere(1,3). By the end of 24 hours after administration, approximately 55-65% is still in the circulation and since it is gradually metabolized, free I_{131} is liberated, and may be taken up by the thyroid. This can be successfully prevented by "blocking" the thyroid with stable iodine in the form of Lugol's solution, 10 drops, 3 times daily for a week, starting at least 24 hours previous to the administration of RIHSA(1). RIHSA was selected as a tracer agent for blood volume determination because it fulfills better than any other substance known at present, the basic criteria as laid down by Keith and Rowntree(2).

Fig. 1 shows the design of the 3-tube Geiger counter constructed for the blood volume determinations. The 3 tubes were held in place on a steel disc by a lucite plate. A thin wall brass tube was soldered in the center to hold

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[*] Supported in part by grants of the Atomic Energy Commission, and the U. S. Public Health Service.

[†] Obtained from Abbott Laboratories.

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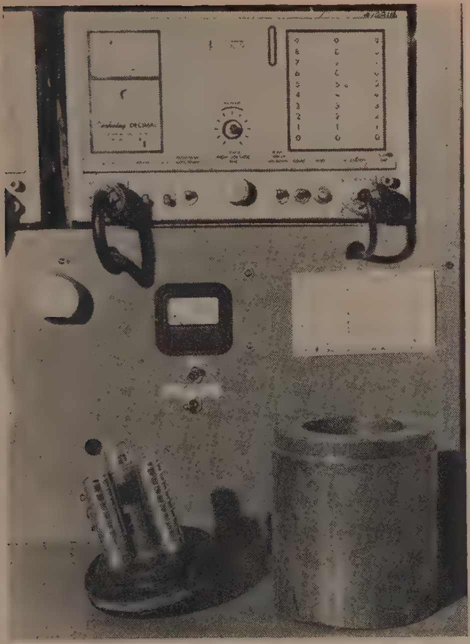

FIG. 1.

the cuvettes. This counter was mounted in the lead cylinder to reduce the background count. A five foot coaxial cable connects the counter to a Berkeley Scaler, Model 1000B, which is controlled by a predetermined time-interval clock. Coleman 6-304A cuvettes which accurately fit the central brass tube of the triple thyrode G-M counter were calibrated to contain 10 ml. Heparin solution was used as an anticoagulant; other anticoagulants have been investigated and found, as will be pointed out later, extremely unsuccessful.

The selection of a counting system was dictated by the requirements of rapid measurement of liquid samples with an accuracy of 1-2%, and a counting efficiency which would make feasible blood volume determinations with the tracer dose containing less than 60 μc of I_{131}. Construction of the counter made possible the direct comparison of this method with dye methods, since the same blood specimen and cuvettes were used for both technics. Thus the counting procedure was analogous to the colorimetric technics. The use of 3 Geiger tubes surrounding the central brass tube (which holds the cuvette) eliminates errors due to positioning the sample. The system was designed to count 10 ml of either blood or a standard "active solution" in a selected cuvette. The size of cuvette and position of the brass tube are such that the first 5 cc and the second 5 cc of solution gave equal contributions to the count. This eliminates, almost completely, errors introduced by sedimentation of the blood if this be allowed to occur. Measurements indicated this error is less than 1%.

Comparison of solutions of I_{131} in water and in blood indicated that the error of absorption, introduced by the change in the medium is less than 1%. In this study aqueous comparison solutions were used instead of making up these solutions with blood as the diluting medium. The sensitivity of the counter was 2820 counts per minute for 1 μc of iodine (point source) in the center of the tube or 2540 counts per minute per μc of I_{131} in 10 ml of solution (National Bureau of Standards distribution of 1-15-51). Efficiency of the counter was 0.11% for iodine in 10 ml of solution. (Not corrected for failure to achieve 4 π geometry). Substitution of bismuth cathode Geiger tubes[‡] of the same size will increase this by a factor approximating 5. Comparison with a multiple anode cup counter[§] indicated this counter would increase the sensitivity by a factor just exceeding 20, and would allow a considerable reduction in the required dose.

Procedure. From every batch of RIHSA delivered, a sterile tracer solution is prepared in such a dilution that 10 cc of it contains 60 μc of I_{131}. Of the tracer solution 1 cc is further diluted to 100 cc of which 10 cc is used as the standard. This standard therefore contains 0.6 μc which will give about 7500 counts per 5 minutes in the triple thyrode counter. Using calibrated syringes, each patient is given 10 cc of the tracer solution intravenously containing from 30-60 μc

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[‡] Constructed for us by Radiation Counter Laboratories.

[§] Type H18-20TR (cup 20) supplied through courtesy of Dr. Herzog of Texas Company, Bellair, Texas.

I_{131} depending on the decay-factor; 10 minutes after administration, blood is withdrawn from the antecubital vein of the opposite side with a heparinized syringe and 10 cc immediately delivered into a selected cuvette. The cuvette is inverted several times to assure homogeneous suspension of the blood corpuscles. It is then put into the triple thyrode counter and counted for 5 minutes. The total blood volume can be calculated by the following formula:

$$\text{TBV} = \frac{\text{Counts/5 min of the standard} \times \text{No. of cc given of tracer solution}}{\text{Dilution of standard} \times \text{counts/5 min of the specimen.}}$$

In order to study the validity of the technic under investigation, T-1824 dye was used simultaneously in patients to study blood volumes. We have also studied plasma volumes with RIHSA technic in a group of patients. This was done by centrifuging about 30 cc of heparinized blood that had been withdrawn through the same venipuncture made for obtaining the 10-minute blood specimen. Centrifugation at 3000 RPM was carried out for 30 minutes and the clear supernatant plasma pipetted into the calibrated cuvette up to the 10 cc mark. The plasma activity is counted and plasma volume calculated in the same manner previously described. From the same blood sample, 2 Wintrobe hematocrit tubes were filled and centrifuged at the same speed together with the plasma specimens. From the blood volume and plasma volume figures obtained, "hematocrit" values can be calculated. These were compared with direct Wintrobe tube hematocrit values in the same patients giving further indices of accuracy.

Results and discussion. A total of 40 cases has been studied up to the time of this report. In all the cases, no effort was made to consider patient's body weight, and height. Since this group was made up of patients with various physiological and pathological conditions, any attempt to express their blood volumes, as determined by the RIHSA technic in terms of body weight will undoubtedly give false indices and therefore be misleading. For purposes of discussion they are separated into 2 groups, the first group consists of 8 cases in whom simultaneous blood volume studies with RIHSA and T-1824 dye were made. The results obtained and the percentage of difference between the two methods are tabulated in Table I.

TABLE I.

Cases	Blood vol I_{131} P technic	Blood vol Evans blue technic	Diff.
1	4120	4920	—16
2	4100	4080	± 0
3	4950	5320	— 7
4	4840	5900	—18
5	4840	5380	—10
6	4915	6250	—20
7	4970	5390	— 8
8	5210	5200	± 0

In case 6, a difference of 20% was observed, this was the highest percentage of difference in this series. In cases 2 and 8, however, virtually no difference existed between the results obtained. It is interesting to notice that almost all figures obtained with the Evans blue technic were higher than those using RIHSA. This finding agrees with those of other workers in the same field(3). It has been suggested that probably Evans blue diffuses out from the vascular system much more quickly than RIHSA. Assuming that all the figures in our series were obtained with the minimum technical error, it seems difficult to evaluate the validity of the technic by merely looking at the percentage of difference listed in Table I. In cases 2 and 8, the results obtained by two technics were almost identical. This is in disagreement with the theory that Evans blue diffuses out from the vascular bed more quickly and should result in a larger volume.

It was then decided to measure plasma volumes which, when calculated against blood volumes of the same patients, would give "hematocrit" values. These values were compared directly with Wintrobe tube readings. Comparison between these two should, for all practical purposes, give more accurate indices. We are justified in carrying out the comparison between the calculated and the actual hematocrit values based upon the following assumptions: (1) That the tracer solution is homogeneously mixed with the circulating blood in a practically closed system by the end of 10 minutes after its injection. This

TABLE II. Statistical Analysis of 30 Cases.

Range in B.V.	1642 to 6390 cc
” ” P.V.	952 ” 3625 cc
” ” calc. HT	23.4 ” 66.7
” ” actual HT	25.5 ” 67
” ” % of diff.	–8 ” +8

Mean diff. (actual-calc. HT) = +0.63%
Std deviation = ±3.95%

Cases	B.V.	Plasma vol	Hemato-crit calc.	Actual HT	% of diff.
5	5820	3100	47	69	–32*
6	4660	3500	25	43	–42*

* See text.

TABLE III.

	Total c/5 min	Absolute c/5 min	%
10 cc plasma + heparin	1577	1009	
10 cc plasma + Na-oxalate	1332	764	
Diff.	245	245	25

was found correct by counting serial blood specimens at 2-minute intervals up to 30 minutes. They were found to contain almost the same amount of radioactivity; (2) That the tracer material is present in plasma only, having no binding or tagging properties on the corpuscles. That is, a given volume of whole blood, actually is composed of 2 compartments, namely a radioactive plasma and a radioactivity-free corpuscular compartment. This was found correct and as will be shown later on, the calculated "hematocrit" and the actual "hematocrit" do actually agree in all cases.

Table II presents the second group of cases in whom the blood volume study was controlled by plasma volume and hematocrit studies. Note cases 5 and 6 in which an extreme difference between the calculated and actual hematocrit was observed. They were the two instances in which sodium oxalate was added as an anticoagulant agent in addition to merely heparinizing the syringes which were used to withdraw blood specimens. The diminishing of calculated hematocrit value was attributed to a false increase in plasma volumes resulted from technical loss of radioactivity from the plasma. Investigation was then started to study this problem and the following case will illustrate the difference in counts of the plasma specimen of the same patient using heparin and sodium oxalate, respectively (Table III).

It was found that approximately 25% of the radioactivity was missing from the oxalated plasma. Since these 2 specimens were treated under the same conditions except for the different anticoagulants used, it was felt that it must be due to Na-oxalate which probably acts on the radioactive compound in such a way that when the blood is centrifuged, the radioactive compound is spun down together with the blood cells. This was subsequently found to be true as indicated by the finding that 10 cc of saline suspension of the packed blood corpuscles from the specimen treated with Na-oxalate contained more radioactivity than that of the specimen treated with heparin. Na-citrate has also been tried and it has a similar effect on the relative distribution of the radioactive material in the centrifuged specimens. This observation is extremely important. It points out the tremendous error that could occur if blood volume determination is to be calculated from hematocrit and plasma volumes with the latter being determined by RIHSA using either Na-oxalate or Na-citrate as anticoagulant. In the technic described by Storaasli *et al.*(3), crystal heparin was used; no mention was made as to whether any other anticoagulants had been tried. However, in our technic for the direct determination of whole blood volume, it makes no difference as to the type of anticoagulant that is employed.

The advantages of direct blood volume determination using RIHSA are worth summarizing: (1) It is a very simple and rapid method; (2) only one blood specimen is required; (3) calculation is minimal, and (4) repeated blood volume determination is possible by giving further doses of RIHSA after determining the residual activity left in the blood stream.

Summary. (1) A clinical laboratory procedure for directly measuring total circulating blood volume using RIHSA as the tracer agent has been described. (2) A series of 40 cases in whom total circulating blood volume

was determined by this technic. (3) The advantages of this technic over the dye method have been discussed.

The authors wish to thank Douglas A. Kohl and Ernest Berkas for their technical assistance.

Received June 1, 1951. P.S.E.B.M., 1951, v77.

Effect of Thyroidectomy on ACTH Content of Rat Adenohypophysis.* (18833)

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The site of formation of ACTH within the adenohypophysis has not yet been conclusively established. Finerty and co-workers (1,2), who observed a transient rise in the number of acidophils after hemiadrenalectomy in the rat, regard this class of cells as the source of ACTH. On the other hand, one of the rat's 2 types of pituitary basophils, the beta cell, has been suggested as the possible producer of the hormone(3). In these studies conclusions were reached by comparing pituitary cell counts with the state of the adrenal gland. It should be recognized, however, that the count of a given pituitary cell type, if due allowance is made for the size and density of granulation of the individual cells, reflects the storage of its secretory material, whereas the condition of the target organ is an index of the level of its governing pituitary principle in the circulation. As storage and discharge of pituitary trophins do not always run parallel, the comparison between the histological structure and the stored amounts of hormones in the pituitary appears to be a more promising approach to the problems of its histophysiology. Since thyroidectomy is known to lead to an extremely conspicuous drop in the number of both the acidophils and the beta cells, while the delta cells become progressively hyperplastic and vacuolated(3), it was of interest to determine how these profound histological changes affect the ACTH stores of the pituitary.

Method. A shipment of 22 young adult male Sprague-Dawley rats (180-200 g) was divided into 2 groups. Nine animals were thyroidectomized, 13 served as controls. The rats received Purina Laboratory Chow and tap water *ad lib.* Thirty days later the members of both groups were sacrificed by decapitation. At this time the thyroidectomized rats weighed 228-256 g (mean 248 g), the intact animals 288-337 g (mean 322 g). The pituitaries of 3 thyroidectomized and 4 control rats were saved for histological examination. The hypophyses of the remaining animals were prepared for a comparative assay of their ACTH content by the ascorbic acid depletion method of Sayers *et al.*(4). In view of the unstableness of ACTH in alkaline solutions(5), lyophilization and subsequent extraction of the glands with saline made alkaline to 0.01 N with sodium hydroxide(6) was abandoned in favor of a technic devised by Sayers(7). The anterior lobes were pooled in a solution of 0.85% sodium chloride made to 10th molar with hydrochloric acid (one pituitary per ml fluid), homogenized and extracted at room temperature. The extracts were transferred to a boiling water bath for 60 minutes,

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TABLE I. Pituitaries of Thyroidectomized Rats Assayed for ACTH Content Against Those of Controls.

	Thyroidectomized donors (6)*		Control donors (9)*	
Dose†	No. recipients	Response	No. recipients	Response
1.25	4	29.2‡ ± 2.7§	5	30.1 ± 1.2
.625	5	17.3 ± 4.1	5	17 ± 1.5
.3125	4	6.4 ± 2.1	4	7.9 ± 1.6
Total	13		14	

Slope of log dose–response curve 39.0 37.1
Significance of diff. between slopes $p = 0.9$

$M = \overline{1}.9727$ $S_M = 0.0548$

ACTH content of thyroidectomy-pituitaries with that of controls at 100% = 94%
Range of potency at 95% level of confidence = 73-118%

* No. of donor rats.
† % donor pituitary per 100 g recipient.
‡ Mean of differences between ascorbic acid concentration in left and right adrenal of individual recipients, expressed as % of concentration in left adrenal.
§ Std error.

centrifuged and the decanted supernatants stored in a deep freeze unit. On the morning of the assay appropriate saline dilutions of the stock solutions were prepared and their pH adjusted to 1.2-2. The extract of the thyroidectomy-pituitaries was assayed against that of the control hypophyses in strict adherence to the design outlined by Burns *et al.*(6). Hypophysectomized male rats (120-160 g) were purchased from the Hormone Assay Laboratories and used 24 ± 3 hours postoperatively. Adrenal ascorbic acid determinations were performed by the method of Roe and Kuether(8). The administration of 0.5 ml per 100 g recipient of a 1:40, 1:80 and 1:60 dilution of the stock solutions was found to be the dose range for which a rectilinear relationship could be established between the log dose and the adrenal ascorbic acid drop. In other words, the hypophysectomized rats received doses equivalent to 1.25, 0.625 and 0.3125% of a donor pituitary, respectively, per 100 g body weight. The analysis of the assay was carried out according to the method of Irwin, as presented by Pugsley(9). Sections from the pituitaries destined for histological examination were stained with aldehyde-fuchsin, hematoxylin, orange and light green. Cell counts were performed in the usual manner.

Results. The assay data are summarized in Table I. It is evident that there was no significant difference between the amounts of ACTH stored in the pituitaries of intact male rats and of animals which had been thyroidectomized for 30 days. As Table II shows, the thyroidectomy-pituitaries were around 25% heavier than those of the controls, which makes the concentration of ACTH in the former about 70% of that found in the control pituitaries.

The regression of the acidophils and the beta cells in the histologically examined hypophyses of the thyroidectomized rats (Table II) is of the same order of magnitude as that previously observed to occur after complete removal of the thyroid, the acidophils being practically absent and the beta cell count around 8% of the control value. The question arises whether or not there is any cogent evidence that the acidophils and the beta cells behaved in a similar fashion in the assayed pituitaries. It has been pointed out by Griesbach and Purves(10) that the degranulation of the acidophils after complete thyroidectomy can be assessed by gross inspection of the pituitary, which becomes distinctly transparent in this condition. All the hypophyses of thyroidectomized rats used in the present

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TABLE II. Pituitary Weights and Cell Counts.

	No. rats	Wt, mg	Acidophil	Beta	Delta	Vacuolated delta
			%			
Controls	4	7.5	44.48	2.16	8.65	.25
Thyroidectomized	3	9.3	.00	.17	37.90	7.43

assay presented this macroscopic alteration to a very pronounced degree and it can be safely concluded that they must have been largely devoid of acidophilic granulation. As to the beta cell count, it has been previously reported(3), and amply confirmed on additional material, that the regression of this cell type is a more sensitive sign of thyroid hormone deficiency than the degranulation of the acidophils. Thus we have no reason to doubt that the ratio between the beta cell counts of the control and the thyroidectomy-pituitaries in the assay material was similar to that seen in the glands subjected to microscopic examination. The same holds true for the hyperplasia of the delta cells.

Discussion. Since the pituitaries of thyroidectomized rats, which contain negligible amounts of beta- and acidophilic granular material, were found to have an ACTH content almost as high as that of normal glands, it has to be concluded that neither the beta nor the acidophilic granule can be the cytological sign of ACTH storage in the rat hypophysis. The failure of the ACTH levels in the pituitary to rise concomitantly with the thyroidectomy-induced increase in the amount of delta-granular material makes a connection between this hormone and the delta granules appear unlikely, without, however, disproving it unequivocally. The fact that ACTH can not be identified with the histologically demonstrable secretory products of acidophils or beta cells does not preclude the possibility that the principle is produced by these cells and stored in the form of submicroscopic particles, but the burden of proof is on those who claim that any single one of the two cell types is the sole source of ACTH, and the evidence so far marshalled in favor of such views can not be regarded as conclusive. The role of the delta cells in the elaboration of ACTH will be the subject of further studies in this laboratory.

The results presented in this paper also indicate that the reduction in the output of ACTH in hypothyroid rats(11,12) is not primarily due to an inability of the pituitary to elaborate adequate amounts of ACTH on account of a "shift" to thyrotrophin production, as suggested by Zarrow and Zarrow(12), but rather to a disturbance in the release of ACTH. The failure of larger than normal quantities of ACTH to accumulate in the thyroidectomy-pituitary, however, indicates that the pace of ACTH production also must be slowed down to such an extent that the physiological balance of elaboration and discharge is maintained. The presence of not significantly diminished pituitary ACTH stores in hypothyroidism explains the findings that thiouracil-treated rats react to epinephrine with a drop in adrenal ascorbic acid(13) and that the stressor effect of starvation leads to marked adrenal enlargement in thyroidectomized rats(14).

Summary. 1. A comparative assay of the pituitary ACTH content of normal and thyroidectomized rats was carried out by means of the adrenal ascorbic acid depletion method in hypophysectomized rats. 2. No significant difference was found between the ACTH stores of the normal and the thyroidectomy-pituitaries. 3. Since the hypophyses of thyroidectomized rats are extremely poor in acidophilic and beta-granular material, it is concluded that neither the granulation of the acidophils nor that of the beta cells can be the morphological equivalent of ACTH storage. 4. The nature of the defective secretion of ACTH in hypothyroidism is briefly discussed.

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Received June 4, 1951. P.S.E.B.M., 1951, v77.

Pharmacology of Nicotine: Antipyretic, Renal and Adrenocorticotrophic Effects. (18834)

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Nicotine has antidiuretic activity in the rat (1,2) and in man(1,3). Derivatives of 3-hydroxycinchoninic acid have been shown to be antidiuretic in the dog(4) and rat(2). In the rat, hypophysectomy abolished this effect of nicotine(1), and it has since been found that partial neurohypophysectomy is sufficient to abolish the antidiuretic effect both of nicotine and 3-hydroxycinchoninic acid derivatives in the rat(2).

Because of this similarity and the concurrent work in this laboratory on various effects of the substituted cinchoninic acids, it became of interest to investigate nicotine with regard to some of these other properties. When the cinchoninic derivatives were assayed for antipyretic activity(5), nicotine was included and found highly active. The temperature studies were therefore extended, and renal and adrenocorticotrophic effects of nicotine were also investigated. These comprise the present report.

In all experiments, solutions of nicotine bitartrate in 0.9% saline were used. Figures given refer to the salt.

Antipyretic. Methods were identical with those previously described(5). The data of Table I permit a quantitative estimation of the antipyretic activity of nicotine in the rat. Based on the earlier study(5), nicotine is 60 times as active as salicylic acid and 10 times as active as the most potent of the cinchoninic acid derivatives. Fig. 1 shows that the antipyretic effect may also be obtained in the rabbit and dog. The effect on the unfevered rat (Table I) is similar to that of acetanilide (6) and 3-hydroxy-2-phenylcinchoninic acid (5); lowering of temperature is observed at a dose 4-6 times greater than that needed for antipyresis. A survey of the literature does not reveal any occasion when nicotine has been given to a fevered animal and the temperature recorded. On the normal animal there are scanty and inconclusive early reports on doses ranging from homeopathic to lethal, where both rises and falls in temperature had been noted. Some of these data were reviewed by Lestschinskaja(7). She found that the rabbit and the guinea pig usually responded to subcutaneous injection of 1 mg/kg with a fall in temperature.

TABLE I. Effect of Nicotine on Temperature of the Rat.

Dose, mg/kg	No. rats	Change in temp. (F) 1 hr after drug
	Yeast fever	
5	4	—3.1
2	4	—2.1
.8	8	—1
.3	4	— .2
Saline	9	0
	Unfevered	
5	6	—1.9
2	3	— .1
.8	3	+ .2
Saline	5	+ .2

Drug was given subcutaneously in approximately 1 ml of solution.

Renal. The antidiuretic effect of nicotine has not previously been reported in the dog, nor has there been any study of creatinine clearance following nicotine. In 5 dogs, the minimum dose for sharp antidiuresis was 0.05-0.20 mg/kg. In these experiments the ani-

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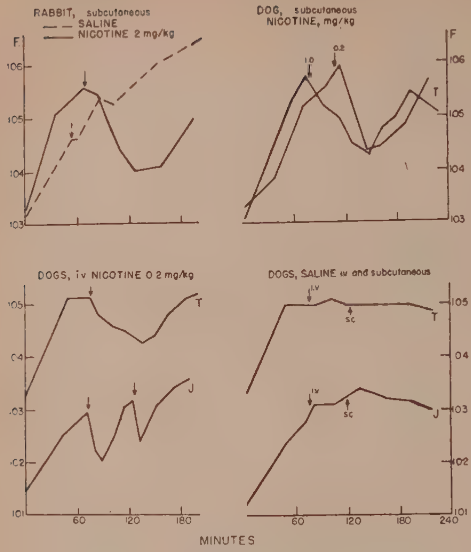

FIG. 1. Effect of nicotine on the febrile rabbit and dog. Fever induced by intravenous Pyromen at 0 time. Arrow at drug or saline. Dogs are identified by letter.

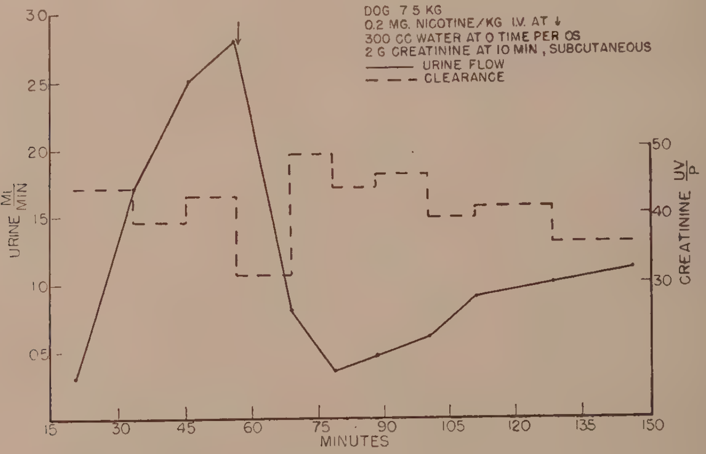

FIG. 2. Effect of nicotine on water diuresis and creatinine clearance in the dog.

mals were fasted for 16-18 hours, 40 ml water per kilogram was given at zero time, and nicotine injected intravenously (approximately 2 ml in 40 seconds) during diuresis. Fig. 2 is a typical curve. This experiment includes creatinine clearance, performed in the usual manner. It may be noted that immediately after nicotine injection there is a transient fall in clearance, which probably corresponds to a vascular effect in this period. Following this, however, and at the lowest points of urine flow, creatinine clearance is essentially normal. Similar results were obtained with 3 other dogs. Creatinine was determined by the Jaffe reaction(8). Nicotine antidiuresis therefore appears to be largely independent of creatinine clearance, which is probably a measure of glomerular filtration in the dog. Like 3-hydroxy-2-phenylcinchoninic acid(4), nicotine appears to increase the tubular reabsorption of water in the dog. In connection with previous studies from this laboratory(9), it was of interest to find whether nicotine affected the renal excretion of phenol red. After the intravenous injection of 6 mg of phenol red and 0.2 mg/kg of nicotine, the amount of dye excreted in 1 and 2 hours was measured. Figures in parenthesis are for phenol red without nicotine. At these times respectively Dog J excreted a total of 2.7 (2.9) and 3.4 (3.8) mg; Dog T a total of 3.6 (3.7) and 4.1 (4.2) mg. Unlike the cinchoninic acid derivatives(9), therefore, nicotine does not appear to inhibit the renal excretion of phenol red.

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TABLE II. Effect of Nicotine on Adrenal Ascorbic Acid in the Rat.

Normal			Hypophysectomized		
	No. rats	mg ascorbic acid/100 g adrenal ± S. E. of mean		No. rats	mg ascorbic acid/100 g adrenal ± S. E. of mean
Saline	13	403 ± 17	Saline	8	329 ± 14
Nicotine 2 mg/kg	6	335 ± 11	Nicotine 4 mg/kg	6	353 ± 13
			ACTH 10 mg/kg	4	199 ± 11

All drugs given subcutaneously 2 hr before removal of adrenals.

Adrenocorticotrophic. Using the same technics and criteria as previously described(10), the effect of low doses of nicotine on adrenal ascorbic acid in normal and hypophysectomized male Sprague-Dawley rats was studied. Table II gives the results. In the intact animal the difference between nicotine and saline is significant ($P < .01$); since hypophysectomy abolishes this effect it appears that nicotine like cinchoninic acid derivatives and a number of other drugs(10), stimulates the adrenal by a pathway which includes the adenohypophysis. In the operated animals, inspection at autopsy with a low power lens revealed total absence of the anterior lobe and a variable amount of neural stalk and lobe.

Discussion. Brief mention may be made of the relation that the above doses bear to the lethal or to those that give prominent side effects. At the range used in the rat there were occasional bouts of irregular breathing 15-30 minutes after injection. No other abnormalities were noted. At 15 mg/kg subcutaneously, tachypnea was observed; at 30-100 mg/kg there were brief convulsions and partial collapse, with recovery. In the dog the order of dose used above (0.1 mg/kg) caused panting and some salivation for about 30-60 seconds after intravenous injection. No signs were seen after subcutaneous injection. Four mg/kg intravenously caused convulsion and collapse, with recovery. The intravenous $L.D._{50}$ in the dog is reported as 5 mg/kg, presumably as free base(11). In the rabbit slight increase in respiratory rate was noted at 1 mg/kg intravenously, but not subcutaneously. The intravenous $L.D._{50}$ in the rabbit is 6.2 mg/kg, presumably as free base(12). In these species therefore the doses used are about 1/25-1/50 of the convulsive or lethal.

It therefore appears that in relatively low doses nicotine shares the antipyretic, antidiuretic, and adrenocorticotrophic effects previously reported for 3-hydroxycinchoninic acid and its derivatives.

Summary. 1. Nicotine lowers the temperature of the fevered rat, rabbit and dog. 2. In addition to the previously described antidiuretic effect of nicotine in rat and man, the present experiments indicate high activity in the dog, and independence of this effect from changes in glomerular filtration rate. Nicotine did not lower the renal excretion of phenol red in the dog. 3. Nicotine lowers the concentration of ascorbic acid in the rat adrenal. This effect is completely abolished by total adenohypophysectomy.

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Received June 4, 1951. P.S.E.B.M., 1951, v77.

Reduction of Urinary Sodium and Potassium of Diabetic Rats Produced by Hypophyseal Growth Hormone.* (18835)

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Growth hormone is known to produce hyperglycemia and glycosuria in normal cats (1) and dogs(2), and to intensify the glycosuria of diabetic rats(3). Growth hormone also reduces urinary nitrogen excretion and promotes gain in weight and deposition of body protein in normal rats(4). Reduction of urinary nitrogen excretion in diabetic rats occurs even when the glycosuria is increased (3). One would anticipate that the deposition of increased amounts of body protein and gain in weight under the influence of growth hormone would be associated with a positive sodium and potassium balance. The negative sodium and potassium balance that is associated with severe, uncontrolled diabetes is well recognized. Insofar as the authors are aware, no observations regarding the effects of growth hormone upon sodium and potassium balance have been reported. It is the purpose of this paper to report the fact that the administration of growth hormone to diabetic rats reduces the urinary excretion of both sodium and potassium even in the face of an increase in glycosuria.

Methods. The diabetic rats used were male animals of the Long-Evans strain that were partially depancreatized when they were from 25 to 32 days old. They were used in the experiments when they were from 120 to 150 days of age, and had an established glycosuria. Each rat was maintained in an individual metabolism cage and 24-hour urine specimens were collected daily for determination of glucose, nitrogen, sodium, and potassium. Glucose was determined as total reducing substances by the Somogyi procedure, nitrogen by the micro-Kjeldahl method, and sodium and potassium by the Perkin-Elmer flame photometer, model 52A, with a lithium internal standard. Four groups of animals with 4 or 5 animals in each group were studied. Two of the groups were maintained on the regular stock diet and two groups received the same diet with an additional 600 mg of potassium chloride added to the diet daily. In most instances the food intake was constant throughout the period of observation, the method previously described(3) being used to control the food intake. In two of the groups during growth hormone injection, some of the animals reduced their food intake slightly, but the maximum reduction in food consumption was 700 mg per rat per day for the treatment period and the total food intake remained in excess of 20 g per rat per day. The growth hormone was administered twice daily intraperitoneally and was prepared by Dr. C. H. Li, according to the previously published method(5).†

Results and discussion. The data for urinary sodium are summarized in Table I. It will be seen that in all groups growth hormone produced a reduction of urinary sodium and that the greatest reduction occurred in Groups I and IV where there was minimal or no reduction respectively in food consumption during the treatment period. The data for the glycosuria are not shown in the table, but the average glycosuria increased in all groups except Group I. One rat in Group I had a considerable reduction in glycosuria while

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* Aided by grants form Research Board of the University of California, and Christine Breon Fund of the School of Medicine.

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† The authors are indebted to Dr. C. H. Li for generous supplies of the hormone.

TABLE I. Effect of Growth Hormone on Urinary Sodium (Sodium Expressed as mg/Rat/Day).

Group		Control period	3 mg GH per day			6 mg GH per day		
		mg of Na	mg of Na	Change	p§	mg of Na	Change	p
I	Stock diet, 5 rats (20 g food daily)	52 ± 2‖ (10)*	44† ± 4 (3)	—8	.05			
II	Stock diet, 4 rats (20 g food daily)	69 ± 2 (10)	66 ± 1 (3)	—3	.50	63 ± 4 (2)	—6	.20
III	High KCl diet, 5 rats (21 g food daily)	57 ± 1 (10)	51‡ ± 4 (3)	—6	.10			
IV	High KCl diet, 5 rats (21 g food daily)	76 ± 1 (10)	65 ± 3 (3)	—11	<.01	64 ± 3 (2)	—12	<.01

* Fig. in parentheses indicates No. of days of observation.

† Food consumption reduced by 0.4 g per day during this period.

‡ Food consumption reduced by 0.7 g per day during this period.

§ From Fisher table of t.

‖ Std dev. $\sigma = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

TABLE II. Effect of Growth Hormone on Urinary Potassium (Potassium Expressed in mg/Rat/Day*)

Group		Control period	3 mg GH per day			6 mg GH per day		
		mg of K	mg of K	Change	p	mg of K	Change	p
I	Stock diet, 5 rats	58 ± 2 (10)	42 ± 3 (3)	—16	<.01			
II	Stock diet, 4 rats	82 ± 1 (10)	68 ± 5 (3)	—14	<.01	67 ± 5 (2)	—15	<.01
III	High KCl diet, 5 rats	382 ± 4 (10)	353 ± 12 (3)	—29	<.01			
IV	High KCl diet, 5 rats	386 ± 2 (10)	366 ± 5 (3)	—20	<.01	368 ± 9 (2)	—18	<.01

* This table arranged as in Table I, and based on same animals; hence food consumption, etc. are the same as in Table I.

receiving growth hormone and, although the other rats had an increase, there was no enhancement of glycosuria for the group as a whole. The average daily increase in glycosuria was 550, 740, and 830 mg per rat per day in Groups II, III, and IV respectively during the administration of 3 mg of growth hormone per rat per day. In Groups II and IV during the administration of 6 mg of growth hormone per day, the average glycosuria increased 510 and 180 mg per day respectively. When the change in glycosuria was compared statistically on the basis of individual animals, the increase was significant in 7 out of 28 cases.

The data for urinary potassium are summarized in Table II. It will be seen that all groups showed a reduction in potassium excretion which in all cases was statistically significant, and it is to be remembered that there was no change in the food consumption during the treatment period in four of these six sets of observations. In all groups there was a marked reduction in urinary nitrogen varying from 73 to 152 mg per rat per day. When compared statistically on an individual animal basis, this was significant in 21 out of 28 cases. It seems very reasonable that the nitrogen and potassium retaining effects of growth hormone are related, in that both potassium and nitrogen are integral intracellular components of protoplasm.

The increase in glycosuria produced by withdrawal of insulin therapy in a diabetic animal is associated with an increase in the urinary excretion of nitrogen, sodium, and potassium. It is remarkable that the increase in glycosuria produced by growth hormone is associated with a reduction in the urinary excretion of nitrogen, sodium, and potassium. It seems unlikely that any contaminants of the growth hormone preparation used could have been responsible for these effects upon electrolyte metabolism. In our hands, adrenocorticotropic hormone causes sodium and potassium loss when it intensifies the glycosuria of diabetic rats, an effect opposite to that produced by growth hormone in these experiments when it enhanced the glycosuria. Posterior pituitary antidiuretic hormone might be expected to have effects upon electrolyte balance, but one would anticipate that they would be in the direction of sodium loss. The growth hormone preparation used in these experiments was assayed for antidiuretic activity by the Ham and Landis(6) procedure and found to be free of any antidiuretic activity whatsoever at a single dose level of 0.5 mg, a daily dose found to produce changes in sodium and potassium balance of normal animals (unpublished observations).

Conclusion. The administration of growth hormone to partially depancreatized diabetic rats produces a reduction in urinary nitrogen, sodium and potassium excretion, although there may be a concomitant increase in glycosuria.

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Received June 8, 1951. P.S.E.B.M., 1951, v77.

Circulating Antibodies in Vitamin-Deficiency States. Pteroylglutamic Acid, Niacin-Tryptophan, Vitamins B_{12}, A, and D Deficiencies.* (18836)

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Previous work in this laboratory has demonstrated a severe impairment of antibody response in pyridoxine and pantothenic acid-deficient rats(1). Riboflavin(1), biotin and thiamine deficiencies(2) had considerably less effect. Later experiments proved conclusively that the absence of pantothenic acid *per se* and not the concomitant inanition was responsible for antibody impairment(3). Recently we have presented evidence which suggests that pantothenic acid and pyridoxine function directly in antibody synthesis(4).

In the present paper, the relationship between vitamins and antibodies has been extended further to include the effect of deficiencies of pteroylglutamic acid, niacin-tryptophan, vit. B_{12}, vit. A, and vit. D upon the antibody response of the rat to the antigenic stimulus of human erythrocytes.

Methods. Ninety-six male weanling albino rats of the Sprague-Dawley strain were distributed into 5 groups as indicated in Table I. The animals were housed individually in wide-meshed, screen-bottom cages and weighed weekly. The composition of the basal diets fed to each group is presented in Table II. These basal diets were fed *ad lib.* throughout the experimental period. Immunization was instituted in the vit. A, B_{12}, and D groups after 4 weeks, in the niacin-tryptophan group after 5 weeks, and in the pteroylglutamic acid group after 9 weeks on experiment. A 10% suspension of washed Group O, Rh positive human erythrocytes in physiological saline was injected intraperitoneally as antigen. An initial dosage of 0.5 ml of the erythrocyte suspension was followed by two 1 ml injections on alternate days. Five days after the final injection, the rats were bled from the

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* This investigation was supported by grants from the National Vitamin Foundation, and Hoffmann-La Roche.

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TABLE I. Summary of Growth Data, Blood Counts, and Bone Ash Analyses.*

Type	No. of rats	Body wt		Blood counts†		% bone ash	
		Initial	Final†	Leucocytes‡	Granulocytes‡	Femurs	Humerii
Pteroylglutamic acid							
Controls	10	39	320	19800	1700	—	—
Deficient	9	38	141	4900	100	—	—
Niacin–tryptophan							
Niacin controls	5	44	208	—	—	—	—
Tryptophan controls	5	44	189	—	—	—	—
Deficient	10	43	66	—	—	—	—
Vit. A, Controls	10	44	172	—	—	—	—
Deficient	15	44	79	—	—	—	—
Vit. B_{12}, Controls	6	45	129	—	—	—	—
Deficient	7	45	90	—	—	—	—
Vit. D, Controls	10	42	86	—	—	34.66	32.26
Deficient	9	43	73	—	—	20.24	23.78

* All values are group averages.

† At time of bleeding for antibody determination.

‡ No. per cmm of blood.

TABLE II. Composition of the Basal Diets.

Component	Pteroyl-glutamic acid†‡	Niacin-tryptophan†§	Vit. A‖¶	Vit. B_{12}†**	Vit. D††
			(% composition)		
Casein "vitamin-free"	25	9	25	—	—
Sucrose	57.77	44.62	60.76	—	—
Hydrogenated vegetable oil	10	—	—	—	—
Corn oil	2	2	—	5	—
Cotton seed oil	—	—	10	—	—
Salts (5)	4	4	4	2	—
Sulfasuxidine	1	—	—	—	—
Choline chloride	.2	.20	.20	.10	—
i-inositol	.03	.03	.03	.02	—
dl-α-tocopherol acetate	.01	.01	.01	.01	—
2-methyl-1, 4-naphthoquinone	.001	.001	.001	.001	—
L (−) cystine	—	.15	—	.30	—
p-aminobenzoic acid	—	—	.01	.025	—
Iodinated casein	—	—	—	.10	—
Corn grits	—	40	—	—	—
Soybean meal (6% fat)	—	—	—	46.22	—
Whole ground yellow corn	—	—	—	46.22	76
Ground gluten	—	—	—	—	20
Calcium carbonate	—	—	—	—	3
Sodium chloride	—	—	—	—	1
			Daily vit. supplementation (γ)*		
Thiamine	40	40	40	160	40
Riboflavin	60	60	60	240	60
Pyridoxine	50	50	50	200	50
Calcium pantothenate	300	150	150	800	150
Niacin	100	—	100	800	100
Biotin	3	1	1	10	1
Pteroylglutamic acid	—	1	1	10	1

* Supplied in daily pill.

† All animals received 2 drops haliver oil weekly *per os* furnishing 3000 U.S.P. units of vit. A and 24 U.S.P. units of vit. D.

‡ 20 μg of pteroylglutamic acid were included in daily vit. pill of controls.

§ Daily vitamin pill of niacin controls contained 150 μg niacin. For tryptophan controls, basal diet was supplemented with 0.4% DL-tryptophan at expense of sucrose.

‖ Each rat received weekly 250 U.S.P. units of crystalline vit. D_3 (Drisdol) *per os* and 10 mg of ascorbic acid by intraperitoneal injection.

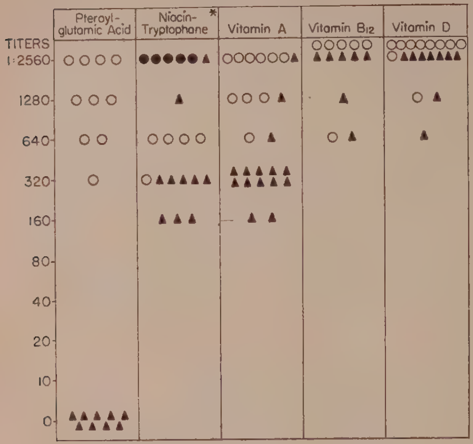
¶ Controls received weekly 2 drops Wesson oil containing 3000 U.S.P. units of vit. A acetate.

** Controls received 0.4 μg of vit. B_{12} on alternate days by intraperitoneal injection.

†† Controls received weekly *per os* 250 U.S.P. units of crystalline vit. D_3 (Drisdol).

TABLE III

INDIVIDUAL HEMAGGLUTININ TITERS



TITERS	Pteroyl-glutamic Acid	Niacin-Tryptophane *	Vitamin A	Vitamin B_{12}	Vitamin D
1:2560	○○○○	●●●●●▲	○○○○○○▲	○○○○○ ▲▲▲▲▲	○○○○○○○○ ○▲▲▲▲▲▲▲
1280	○○○	▲	○○○▲	▲	○▲
640	○○	○○○○	○▲	○▲	▲
320	○	○▲▲▲▲▲	▲▲▲▲▲ ▲▲▲▲▲		
160		▲▲▲	▲▲		
80					
40					
20					
10					
0	▲▲▲▲▲ ▲▲▲▲				

○ - *Control animals*
● - *Tryptophane controls*
▲ - *Deficient animals*
* - *In this group the clear circles represent the niacin controls*

heart, under ether anesthesia and the sera tested for agglutinin titers as described previously(1). Total leucocyte determinations and differential counts were carried out in the animals of the pteroylglutamic acid group in accordance with standard procedures. Two hundred cells were counted in the differential determinations. Bone ash analyses of the femurs and humerii of the animals of the vitamin D group were made at the termination of the experiment.

Results. The individual hemagglutinin titers are recorded in Table III. It is evident that a severe impairment of antibody response occurred in pteroylglutamic acid-deficient rats. Thus, none of these animals possessed a measurable agglutinin titer. The decreased growth rate, leucocyte and granulocyte counts constitute evidence for the development of a pteroylglutamic acid deficiency state (Table I).

The results of the niacin-tryptophan experiment demonstrated that supplementation of the basal diet (deficient in both niacin and tryptophan) with either niacin or tryptophan produced a marked growth response (Table I). In contrast, however, a *normal* antibody response was obtained only in those animals receiving added tryptophan (Table III). Thus, the serum antibody content of the niacin controls did not differ significantly from that observed in the deficient animals. It is also evident that a niacin-tryptophan deficiency produced only a relatively moderate impairment of antibody response.

It may be further seen in Table III that a significant decrease in antibody levels was observed in vitamin A-deficient rats. The magnitude of this decrease, however, was not as great as that observed in pteroylglutamic acid or pantothenic acid-deficient rats, where, as a general rule the serum antibody content following the same antigenic stimulus is not detectable by our methods(1,3). Characteristic vitamin A-deficiency symptoms were observed in the animals not receiving the vitamin A supplementation.

In contrast to the above observations, no significant difference was noted between the antibody titers of vit. B_{12} or vit. D-deficient rats and those of their respective controls. A marked decrease in the percentage of bone ash was observed in the rats of the vit. D group not receiving added vit. D (Table I).

Discussion. The results of the present experiment clearly demonstrate that a severe impairment of antibody response occurred in pteroylglutamic acid-deficient rats. Similar findings were reported by Little *et al.*(6) in chicks which can be made pteroylglutamic acid-deficient without recourse to sulfa drugs. It is well recognized that a pteroylglutamic acid deficiency produces a leucopenic state. Since lymphocytes may be the site of antibody formation(7-9) it is conceivable that the suppression of circulating antibodies observed in pteroylglutamic acid-deficient rats could be attributed to the parallel development of the leucopenia. Preliminary curative experiments

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in which massive doses of pteroylglutamic acid were administered to previously immunized pteroylglutamic acid-deficient rats resulted in: (a) an immediate and sustained growth response, (b) a pronounced rise in the number of leucocytes within 7 days, and (c) no significant increase in antibody titers within 3 weeks. Thus, the leucopoietic response was not paralleled by an increase in antibody synthesis. It is of interest to note that the delay in antibody response observed in this experiment is quite similar to that following pantothenic acid or pyridoxine administration to previously immunized deficient animals(4).

The pronounced effect of a pteroylglutamic acid deficiency upon antibody response is comparable with that previously noted in a pantothenic acid deficiency state(1,3). The observation of Wright and Welch(10) that the utilization of pantothenic acid may be dependent upon the presence of pteroylglutamic acid suggests that the action of pteroylglutamic acid upon antibody synthesis may be mediated through its effect upon pantothenic acid metabolism. Further experimentation will be required to determine whether pteroylglutamic acid plays a direct or indirect role in the processes of antibody fabrication.

In agreement with previous observations (11-13), supplementation of the low niacin-tryptophan diet with either niacin or tryptophan produced a marked growth response. However, a normal antibody response was obtained only in those animals receiving added tryptophan. The efficacy of tryptophan in promoting both growth and antibody synthesis is explicable in terms of its ability to serve as a direct precursor of niacin(14) or niacin derivatives as well as its essential function in protein synthesis. On the other hand, the mechanism through which niacin affects growth under these conditions is not completely understood. In this connection, Krehl *et al.*(12) have observed that niacin improves the utilization of tryptophan. Niacin may also exert a "sparing" effect upon the requirement for tryptophan. Whatever the mechanism, it seems clear that niacin alone cannot satisfy the requirement for antibody synthesis on this basal diet. With the administration of niacin, it would appear that tryptophan is the limiting factor for antibody formation although apparently sufficient amounts of this amino acid are made available under these circumstances for growth purposes. The production of a more pronounced tryptophan deficiency state by employing a diet completely devoid of tryptophan would aid in clarifying the role of this compound in antibody synthesis. In the course of our studies on the relationship of nutritional state to antibody formation, we have occasionally encountered similar instances where a diet may be adequate for growth purposes and yet not for antibody synthesis. Thus, it would seem that the process of antibody synthesis is a more sensitive criterion of dietary adequacy than that of growth and, as such, may be used to advantage in the evaluation of nutritional state.

The role of vit. A in influencing susceptibility to infection has been the subject of numerous investigations(15-18). Studies (19-21) designed to determine the ability of vit. A-deficient animals to form antibodies following antigen administration have shown no impairment of this function. In the present experiments, however, the antibody titers of vit. A-deficient rats were definitely lower than those of control animals. The decrease, however,

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was of a lower order of magnitude than that encountered in pantothenic acid, pyridoxine (1), and pteroylglutamic acid deficiencies. It seems possible that the increased susceptibility of vit. A-deficient animals to disease processes may be attributed in part to their decreased antibody-synthesizing ability.

With respect to vit. D-deficiency, it is generally agreed that rachitic animals are no more susceptible to infection than controls receiving adequate doses of this vitamin(15). In addition, studies(20,21) of the ability of rachitic animals to produce antibodies following antigen injection have indicated that there is no impairment of this function. In agreement with these observations we found no significant difference between antibody levels of vit. D-deficient rats and those of control animals.

The experiments reported in this paper complete an extensive study of the effect of the following deficiency states upon the antibody response of the albino rats (Sprague-Dawley strain) to the antigenic stimulus of Group O, Rh positive human erythrocytes: pantothenic acid, pyridoxine, riboflavin(1), biotin, thiamine(2), pteroylglutamic acid, niacin-tryptophan, vit. B_{12}, vit. A, and vit. D. Identical immunological procedures were employed throughout these investigations. From our results it is possible to classify roughly the effects of these deficiencies upon circulating antibodies into 3 groups as follows: Group I. Severe impairment of antibody response—pantothenic acid, pyridoxine, and pteroylglutamic acid deficiencies. Group II. Moderate impairment of antibody response—riboflavin, thiamine, biotin, vit. A, and niacin-tryptophan deficiencies. Group III. No impairment of antibody response—vit. B_{12}, and vit. D deficiencies.

Summary. 1. Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in pteroylglutamic acid, niacin-tryptophan, vit. B_{12}, vit. A, vit. D-deficient rats and their respective controls. 2. Severe impairment of antibody response was observed in the pteroylglutamic acid-deficient rats. Moderate impairment was demonstrated in vit. A and niacin-tryptophan deficiencies. Vit. B_{12} and vit. D deficiencies had no effect on antibody formation. 3. A rough classification of the effect of 10 deficiency states upon antibody production is presented.

Iodinated casein which contains 0.7% 1-thyroxine was supplied through the courtesy of Dr. George O. Kohler of the Cerophyl Laboratories, Inc.

The authors wish to thank Mr. D. B. Henis and Mr. G. Marco for technical assistance during the course of this investigation.

Received June 11, 1951. P.S.E.B.M., 1951, v77.

Relative Effectiveness of Pantothenic Acid and Panthenol in Stimulating Antibody Response of Pantothenic Acid-Deficient Rats.[*] (18837)

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Panthenol, (α,γ-dihydroxy-N-(-3-hydroxypropyl)-β,β-dimethyl butyramide) the alcohol analogue of pantothenic acid, was demonstrated by Pfaltz(1) to be approximately as effective as pantothenic acid in enhancing growth and curing pantothenic acid deficiency symptoms of the rat. Later Burlet(2) showed that panthenol was oxidatively converted to

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[*] This investigation was supported by grants from the National Vitamin Foundation, and Hoffmann-La Roche, Inc.

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TABLE I. Relative Effectiveness of Pantothenic Acid and Panthenol in Stimulating Antibody Response of Pantothenic Acid Deficient Rats.

Pantothenic acid deficient			Pantothenic acid controls			Panthenol controls		
Wt (g)			Wt (g)			Wt (g)		
Initial	Final*	Titer	Initial	Final*	Titer	Initial	Final*	Titer
39	85	0	47	261	1:5120	46	247	1:5120
44	74	0	47	257	5120	48	278	2560
48	115	0	46	260	5120	47	278	2560
42	78	0	48	288	5120	46	247	5120
40	78	0	44	262	5120	44	248	5120
41	85	0	48	287	2560	47	255	5120
44	74	0	44	267	5120	45	256	5120
41	110	0	46	270	2560	48	284	5120
44	73	0	46	260	5120	46	254	2560
36	61	0	45	261	2560	44	246	5120
40	77	0						
41	83	0						
34	79	0						
41	89	0						
37	58	0						
47	78	0						
Avg 41	81	0	46	267	1:4352	46	259	1:4352

* At time of bleeding.

pantothenic acid in both rats and humans. Recently, Rubin and his co-workers(3,4) on the basis of urinary excretion studies have concluded that panthenol is more available to rats and humans than calcium pantothenate when fed at high dosage levels. On the other hand at the lowest level tested, the availability of the two substances was found to be essentially the same.

Since in the course of our studies on the relationship of nutrition to immune response, we have occasionally noted that the process of antibody synthesis is a more sensitive criterion of dietary adequacy than that of growth or symptomology(5), it seemed of interest to determine whether panthenol is as effective as calcium pantothenate in preventing the observed suppression of circulating antibodies in pantothenic acid-deficient rats(6,7).

Experimental. Thirty-six male weanling albino rats were distributed into 3 groups as indicated in Table I. Except for the omission of sulfasuxidine, the basal diet was identical with that employed in the preceding paper(5) for the production of a pteroylglutamic acid deficiency. All animals received a daily vitamin pill supplying the following vitamins: thiamine, 40 γ; riboflavin, 60 γ; pyridoxine, 50 γ; niacin, 100 γ; biotin, 1 γ; and pteroylglutamic acid, 1 γ. The pantothenic acid-deficient rats received no further vitamin supplement while the pantothenic acid and panthenol controls received a daily intraperitoneal injection of 100 γ of calcium pantothenate and a molar equivalent dosage of panthenol (86 γ), respectively. All animals were fed the basal diet *ad libitum*. Immunization was instituted during the 5th week of the experiment. The antigen, immunization procedure, and the determination of serum antibody titers were identical with those described in the preceding paper(5).

Results. The data obtained are presented in Table I where it may be seen that panthenol was equally as effective as pantothenic acid in promoting both normal growth and attainment of a high serum antibody concentration. These results are in accord with previous studies by other workers(1,2,4) demonstrating the quantitative conversion of panthenol into pantothenic acid by the rat.

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Summary. 1. Panthenol was found to be equally as effective as pantothenic acid in promoting antibody synthesis in pantothenic acid-deficient rats.

We are indebted to Dr. E. L. Sevringhaus of Hoffmann-La Roche, for the generous supply of panthenol.

Received June 11, 1951. P.S.E.B.M., 1951, v77.

Experimental Production of Post-Tonsillectomy Bulbar Poliomyelitis.*

(18838)

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Poliomyelitis occurring within 30 days after tonsillectomy is predominantly bulbar(1). When compared with the incidence of the bulbar form in patients who have not had the operation within the same period of time, the difference is greater than can be explained on the basis of pure chance and indicates a causal relationship between operation and bulbar localization. This is particularly true of children, as shown by the two examples from the literature given in Table I.

In human bulbar poliomyelitis, including the post-tonsillectomy cases, the muscles of deglutition and phonation, supplied by the X cranial nerve and the nucleus ambiguus, are practically always involved, the facial muscles (N.VII) in about half, and those supplied by the V and XII nerves in lesser numbers(4). The nuclei of supply in all these are in the pons or medulla. Post-tonsillectomy bulbar poliomyelitis occurs almost exclusively during epidemics of the disease, and the onset of symptoms according to Aycock's(1) compilation nearly always occurs 6-24 days after operation, with the peak at 14-16 days. These figures agree closely with the incidence period of poliomyelitis in general, suggesting that virus may be introduced at the time of operation. Only rare cases have been reported in which the onset was less than 4 days after operation. Previous attempts(5-7) to reproduce experimentally the sequence of bulbar paralysis after tonsillectomy have nearly always failed, paralysis when produced usually having been spinal in type. In most of these studies, notably that of von Magnus and Melnick(7) exposure of the throat to the virus has been made *after* rather than before operation. Unpublished observations in our laboratory have shown that in order consistently to produce infection in divided nerve it is necessary to apply virus to the central cut end before it is sealed off by blood.

TABLE I. Bulbar Poliomyelitis in Poliomyelitis Cases With and Without Recent Tonsillectomy.

	Poliomyelitis			
	Bulbar	Other forms	% bulbar	P*
A.(2) 0-10 years				
Tonsillectomy	24	12	66.7	
No tonsillectomy	771	6063	11.6	<.001
B.(3) 3-7 years				
Tonsillectomy	12	4	75	
No tonsillectomy	96	395	19.5	<.001

* From chi square of differences between bulbar and other forms. In both series P indicates that probability of differences occurring by chance are less than 1 in 1000.

In the present study we have explored two hypotheses: (1) that virus already in the pharynx is directly introduced into the peritonsillar musculature, which is unavoidably traumatized during the human operation, and

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* Aided by a grant from the National Foundation for Infantile Paralysis.

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TABLE II. Oropharyngeal Swabbing of Virus With and Without Tonsillectomy, Compared With Tonsillectomy After Intrathalamic Inoculation Without Swabbing.

	Oropharyngeal swabbing		Intrathalamic inoculation and tonsillectomy
	Tonsillectomy Series 1	No tonsillectomy Controls	Series 2
Animals exposed	7	7	6
Poliomyelitis	7	5	6
Onset (days)	7-10	8-10	5 (2)*
Bulbar paralysis	7	1	5†
Spinal paralysis	3	4‡	5
Cervical	3	4	5
Thoracic	0	?§	5
Lumbar	0	3	5

* 5 days after inoculation, 2 days after tonsillectomy.
† 1 animal found dead, not included in list of paralyses, had typical lesions.
‡ 1 animal, not included here, had typical lesions.
§ Not specifically noted.

hence into its supplying nerves; (2) that localized traumatic stresses to these muscles tend to localize paralysis in them presumably by making their supplying nerve centers in the medulla more vulnerable to the effects of poliomyelitic infection already present in the central nervous system.

In the test experiments, the tonsils were removed by enucleation with curved scissors; in Series 1, immediately after the application of virus to the pharynx; in Series 2, 3 days after inoculation of virus into the thalamus (no pharyngeal application of virus). In the control series, virus was gently swabbed on the surfaces of the mouth and pharynx but no tonsillectomy was performed. In all experiments a 17% suspension of the Wis '45 strain (Brunnhilde type) of poliomyelitis virus, PD_{50} 4.9, was used. All monkeys were cynomolgus (*M. irus*), previously prepared by division of the olfactory tracts to preclude entry of infection through the olfactory nerves. The results are shown in Table II.

It will be noted that in both series, bulbar poliomyelitis resulted, with the differences that in Series 1, the incubation period was 7-10 days after operation while in Series 2, the incubation period was 5 days after intrathalamic inoculation[†] and 2 days (or slightly less) after tonsillectomy; and that in Series 1 paralysis was limited to the bulbar and (in 3 animals) the cervical innervations while in Series 2, the entire cord, in addition to the bulbar centers, was paralytically involved.

The results with pretonsillectomy exposure of the pharynx to virus (Series 1) appear to correspond, in terms of incubation period and of distribution of paralysis, to the majority of human post-tonsillectomy cases(1) better than those of Series 2, in which tonsillectomy was performed after infection had become established in the CNS. The rapidity with which paralysis followed tonsillectomy in the latter, as well as its relative extent and severity, strongly suggests that the physical stress of the operation itself greatly enhances the speed, severity and, to some extent, the localization of the infectious process. A comparable human situation may be envisioned in those rare cases where bulbar or bulbospinal paralyses begin three days or less after tonsillectomy.

As regards the commoner type of human post-tonsillectomy poliomyelitis with its longer incubation period and paralyses primarily bulbar or bulbocervical, it is conceivable that the situation may be comparable with our experimental Series 1 in that the virus is being excreted on the pharyngeal surfaces but has not yet attacked the CNS. In other experiments(8) we have shown this excretory phenomenon to occur while infection is limited to certain peripheral ganglia, such as the gasserian and nodose. Assuming the same condition to hold in man during the early, presymptomatic period or in cases that might end without symptoms in the absence of operation, an opportunity would be open for direct introduction of virus into the peritonsillar tissues before the CNS is infected.

[†] In 75 cynomolgus monkeys, used in other studies, the average incubation period after intrathalamic inoculation of Wis '45 virus was 8.7 days; primary bulbar and bulbospinal paralyses occurred in 17.3%, and primary spinal paralyses in 82.7%.

8. Faber, H. K., Silverberg, R. J., Luz, L. A., and Dong, L., *J. Exp. Med.*, 1950, v92, 571.

This possibility suggested a supplementary study of the preventive effects of local antisepsis in the throat. Six cynomolgus monkeys were subjected to the same procedure as in Series 1, excepting that 2% tincture of iodine was applied to the throat one-half minute after swabbing with virus and one minute before tonsillectomy. All these animals remained well.

Further exploration of this procedure is required before its meaning and implications can be properly evaluated. Although such pre-operative antisepsis appears to be harmless, no assurance of its preventive value against poliomyelitis in human beings can be offered.

Summary and conclusions. 1. Application of poliomyelitis virus to the pharynx of cynomolgus monkeys, followed by enucleation of the tonsils was regularly followed after 7-10 days by primary bulbar paralysis, accompanied less often by high spinal paralysis. This effect was probably due to introduction of virus into the innervations of the peritonsillar tissues, notably muscle. 2. Intrathalamic inoculation of virus followed 3 days later by tonsillectomy was regularly followed within 2 more days by bulbospinal paralysis in which all levels of the cord were involved. This effect probably indicates that trauma increases the vulnerability of nerve cells to infection already present in the central nervous system. 3. After oropharyngeal swabbing with virus and no tonsillectomy, spinal paralysis usually resulted, with bulbar manifestations in only one instance. 4. With pharyngeal application of virus followed by 2% tincture of iodine and then by tonsillectomy, all 6 animals remained well.

Received June 12, 1951. P.S.E.B.M., 1951, v77.

In vitro Action of Cortisone on Vasoconstriction Due to Histamine.* (18839)

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Two questions prompted the present study. Does cortisone alter the response of isolated smooth muscle to histamine? If it does, what mechanism is involved? The experimental evidence suggesting the participation of the hormones of the adrenal cortex in the metabolism of histamine has recently been reviewed by Rose(1) and need be mentioned only briefly. Adrenalectomized animals have been found to have a markedly diminished resistance to histamine; an increased tissue content of the compound as well as a diminished capacity of the tissues *in vivo*(2) and *in vitro* (3,4) to destroy or inactivate histamine. While these changes are partially reversible by DOCA, adrenal cortical extract (which contains cortisone as well as many other compounds) will restore the metabolism of histamine by the adrenalectomized animal to an essentially normal state(1).

Experimental procedure. An angioplethysmokymographic technic(5,6) was used to

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* These studies were aided by research grants from the Masonic Foundation for Medical Research and Human Welfare and the Hochstetter Fund.

1. Rose, B., *An. Rev. Med.*, 1951, v2, 155.

2. Rose, B., *Am. J. Physiol.*, 1939, v127, 780.

3. Rose, B., Thesis, McGill Univ., Montreal, Canada, 1939.

4. Karady, S., Rose, B., Browne, J. S. L., *Am. J. Physiol.*, 1940, v130, 539.

5. Smith, D. J., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 449.

6. Smith, D. J., Syverton, J. T., Coxe, J. W., *Circulation*, In press.

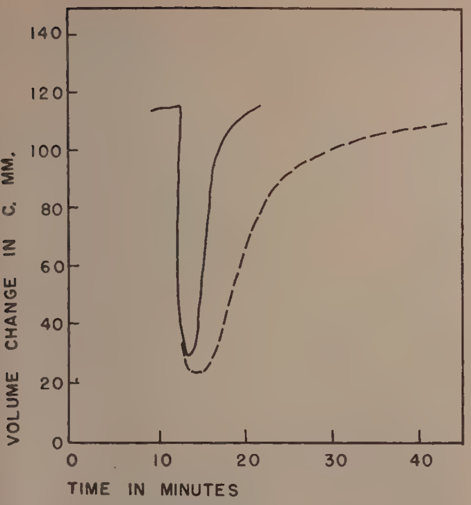

FIG. 1. Exp. #1241. Swine carotid artery. Vasoconstriction to 50 γ test doses of acetylcholine. (Control reaction, ———; Physostigmine 1:1,000,000, – – – –). Note prolongation of "curve of recovery" following inactivation of cholinesterase.

assess the modifying action of cortisone on the vasoconstriction of isolated surviving blood vessels caused by histamine. In this procedure an artery is mounted in a simple plethysmograph and perfused with Tyrode's solution for several hours. A continuous record is made of the changes of volume of the vessel during perfusion. The arteries were stimulated to constrict by injecting test doses (usually 50 γ) of histamine or acetylcholine into the perfusate in such a manner that the stimulating drug flowed through the artery in 12-15 seconds(7). Hence the constriction of the vessel and its recovery occurred in the absence of any detectable portion of the stimulus in the perfusate. The modifying action of cortisone[†] and control substances was determined by adding these compounds to the perfusates in the stated concentrations. The cortisone (free alcohol) was first dissolved in propylene glycol in a concentration of 2 mg per ml. Propylene glycol alone did not modify the reactivity of the arteries in the concentrations used.

Results. The angioplethysmokymographic technic produces time-volume curves (Fig. 1) indicating arterial vasoconstriction to specific stimuli. The "curve of constriction" (decrease in volume when the vessel constricts) is a function of the amount of vascular smooth muscle which is stimulated to contract. Previous studies have shown that this can be eliminated or reduced significantly by typical blocking agents(6). The "curve of recovery" (the increase in volume of the vessel as it dilates back to its original baseline) is primarily a function of the enzyme systems which destroy or inactivate the stimulating agent. The influence of cholinesterase on vasoconstriction due to acetylcholine is demonstrated in Fig. 1.

Preliminary observations of the action of cortisone indicated that it did not act as a physiological antagonist of histamine and furthermore, that it had little or no blocking action, there being no significant change in the "curve of constriction." The swine carotid artery responds to repeated test doses of histamine (50 γ) with reactions which have a similar "curve of constriction," but in which the "curve of recovery" becomes increasingly prolonged,[‡] although the response to similar multiple test doses of acetylcholine over the same time period are unchanged. This rather specific prolongation of the recovery from vasoconstriction due to histamine provided the test object necessary for this study. The arteries were perfused for 2-4 hours and subjected to repeated test doses of histamine. When the recovery phase became significantly prolonged, cortisone (1:2,000,000 to 2:1,000,000 of the free alcohol) was added to the perfusate and the response to histamine was again recorded. A final control period using corti-

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7. Emmel, V., Smith, D. J., To be published.

[†] Cortisone (free alcohol) was kindly supplied by Augustus Gibson, of Merck and Co., Rahway, N. J.

[‡] During the winter months, prolongation of vasoconstriction due to histamine becomes evident after 3 test doses and marked in the fifth reaction (average of 61 arteries) while during summer months, prolongation of reaction does not become evident until after 9 test doses (average of 34 arteries) and rarely becomes as marked as in winter killed animals. These observations on the seasonal variation in this reaction extend from April 1949 to May 1951 and are being continued(8).

8. Smith, D. J., To be published.

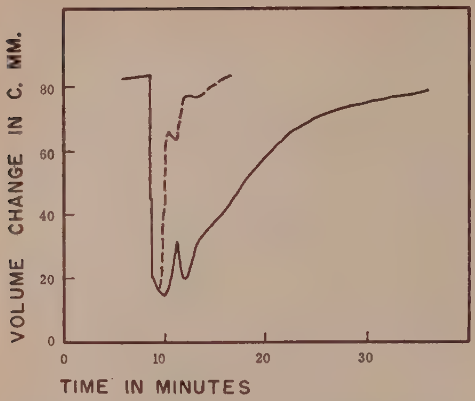

FIG. 2. Exp. #1324. Swine carotid artery. Vasoconstriction to 50 γ test doses of histamine. (Control reaction, ———; cortisone 2:1,000,000, – – –). Note marked acceleration of recovery of vessel in presence of cortisone.

sone-free perfusate was usually run.

Thirty-one experiments of this type using cortisone have been accomplished. In each instance at concentrations of 1 or 2 parts of cortisone per million, the recovery of the vessel from vasoconstriction due to histamine was accelerated (Fig. 2). In a concentration of 1 part of cortisone per 2 million, negligible effect was observed. Control observations using cholesterol in propylene glycol and propylene glycol alone were without effect on the histamine response. Three segments of a single artery were frequently examined simultaneously, each being injected at the same time. One vessel would be retained as a control while the other two were perfused with cortisone. These control vessels in no case showed the accelerated recovery from histamine shown by those vessels exposed to cortisone.

One further observation is of interest. The swine carotid artery constricts when the temperature is raised to 42°C. Cortisone causes the vessel to dilate when it is constricted by temperature.

Discussion. Our results indicate that cortisone (in concentrations of 1 part per million or greater) accelerates the recovery of the vascular smooth muscle from vasoconstriction due to histamine. The mechanism of this accelerated recovery is not completely defined by these studies. In our experience the enzyme systems inactivating the stimulating agent are a major factor in determining the form of the recovery curve. Hence it seems probable that cortisone may modify the histamine inactivating enzyme systems of smooth muscle to produce the accelerated recovery of the tissue observed in this study. This is in agreement with the results of Rose(3) and Karady, Rose and Browne(4), who demonstrated that the decreased histaminase content of the lung of the adrenalectomized rat could be restored to normal by adequate amounts of adrenal cortical extract. The question may be raised, Is this prolongation of the histamine reaction (which is reversed by cortisone) a specific change in the histamine inactivating enzymes or is this a nonspecific deterioration of the tissue *in vitro?* Two facts suggest the latter is not the case. The response to multiple doses of acetylcholine remains constant while concomitant doses of histamine produce gradually lengthening reactions. Secondly, the marked and consistent seasonal variation in the deterioration of the histamine response cannot be explained on the basis of tissue damage caused by the experimental procedure.

Summary. 1. Cortisone accelerates the recovery of vascular smooth muscle from vasoconstriction due to histamine. 2. This probably represents an action of cortisone on the histamine inactivating enzyme systems.

The valued technical assistance of Mr. Clarence P. Bray is hereby gratefully acknowledged.

Received June 15, 1951. P.S.E.B.M., 1951, v77.

Measurement of Erythroid Mitotic Activity in Bone Marrow by Use of Colchicine.* (18840)

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In order to establish the effectiveness of an erythropoietic stimulus, one conventionally measures the concentration or total volume of erythrocytes, the percentage of reticulocytes, or degree of bone marrow hyperplasia. The values obtained indicate the end products of erythropoietic stimulation but do not yield more than inferential information concerning the dynamic erythropoietic process. Since it is generally agreed that new red blood cells arise ultimately from mitotic division of the erythroid elements of the marrow, it is probable that early evidence of erythropoietic stimulation would be found in marrow mitotic activity. Unfortunately, very few mitotic figures are found even in stimulated bone marrow because of their transient nature. The mitotic arresting property of colchicine has been well known since 1934(1). The arrest of marrow cells, in addition to other active cells, has been described(2-5). In an attempt to measure empirically the functional (mitotic) state of erythroid cells, the marrow of normal and erythropoietically stimulated rats after treatment with colchicine was examined.

Methods. Adult, albino rats of both sexes weighing from 135 to 220 g were used in this study. Colchicine (S. B. Penick and Co.) in aqueous solution was injected subcutaneously in doses from 0.01 to 0.20 mg per 100 g of body weight. Injections were made between 9:00 and 11:00 a.m. to avoid any irregularities produced by the often suggested diurnal variations in mitotic activity. From 1 to 8 hours after the injections, animals were sacrificed with chloroform, partially exsanguinated and the femurs removed. Marrow from the proximal end of the femur was suspended in homologous serum, smeared on slides, fixed in Helley's Fluid and stained with Hemotoxylin-Eosin-Azur II(6).

Results. Many easily recognized mitotic figures were evident in the cells of both erythroid and granulocyte series of marrow treated at dosage levels greater than 0.01 mg per 100 g body weight and duration greater than 1 hour. Although ample evidence was available of intense mitotic activity in all members of the granulocyte series, only the erythroid cells were considered in this study. To quantitate the evidence of mitotic division, 1000 or more nucleated marrow cells of all types were counted and the number of erythroid cells in mitosis was expressed in terms of percentage. The great bulk of dividing erythroid cells were early normoblasts and late erythroblasts and were found in prophase. The basophilic or early erythroblasts blocked in metaphase represented a relatively small number.

Concentration and duration. To measure the influence of dosage level, a total of 32 rats were given from 0.01 to 0.20 mg per 100 g of body weight. Five hours elapsed in this group between the time of injection and sacrifice. It will be noted in Fig. 1 that 0.01 mg produced no change in the mitotic percentage from that of the water injected control. The maximal response was obtained with 0.10 and 0.15 mg and a decrease became apparent at 0.20 mg. The influence of the time between injection and sacrifice was examined and the results are presented in Fig. 2. A total of 55 rats were given 0.01, 0.05 and 0.10 mg per

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* Part of this work appeared in *Fed. Proc.*, 1951, v10, 54.

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2. Dixon, W. E., and Malden, W., *J. Physiol.*, 1908, v37, 50.
3. Balduini, M. and Bertolotti, G., *Haematologica*, 1948, v31, 65.
4. Dustin, P., Jr., *Le Sang*, 1950, v21, 297.
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6. Jones, R. McL., in *McClung's Handbook of Microscopial Technic*, New York, 1950, 236.
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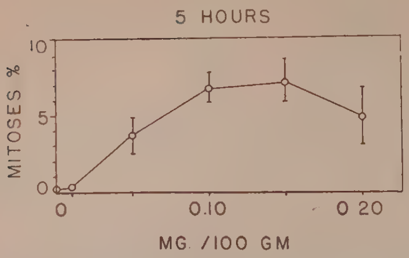

FIG. 1. Influence of increasing dosage of colchicine on erythroid mitotic percentage of rat marrow. Five hr elapsed between injection and marrow sampling.

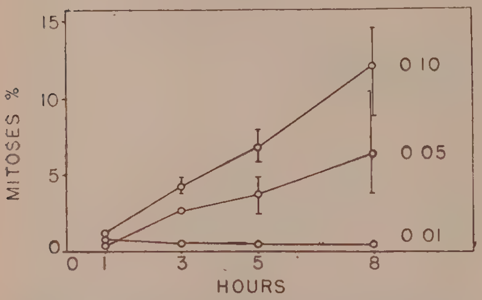

FIG. 2. Influence of increasing time between injection of colchicine and marrow sampling on erythroid mitotic percentage dosage levels expressed in mg per 100 g body wt.

100 g and allowed 1, 3, 5 and 8 hours duration. At the lowest dose, no elevation of the mitotic percentage occurred above that of the water injected control regardless of duration. At both 0.05 and 0.10 mg levels an increase in mitotic percentage occurred with duration to 8 hours. In a few additional measurements made at the 0.10 mg dosage level after 24 hours duration, the mitotic percentage was reduced to that of the water injected control. It may be argued that these changes in mitotic percentage of erythroid cells are a result of either an absolute or relative increase in *total* erythroid elements. To this end, the percentage of total nucleated marrow cells which were erythroid was determined in doses of 0 to 0.20 mg with 5 hours duration. No appreciable change in the erythroid percentage representation was evident in any instance.

Erythropoietic stimuli. If the arrest of erythroid mitoses as demonstrated, measures empirically physiological activity and not merely a pharmacological artifact, the application of a proven erythropoietic stimulus should provoke an increase in the mitotic percentage of marrow above that of the unstimulated rat. All animals were given the standard dose of 0.05 mg colchicine per 100 g body weight and 5 hours elapsed between injection and sacrifice. The erythroid mitotic percentage of 14 normal rats given the standard dose was 2.5 to 4.8%, averaging 3.6%. Fifteen rats were bled by cardiac puncture 2 cc/100 g body weight. One to 3 days after the hemorrhage, the standard dose of colchicine was administered and the mitotic percentage after 5 hours was found to range from 3.8 to 12.0% with an average of 6.9%. These rats had lowered hematocrit values as a result of the bleeding but had not at that time shown a marked reticulocyte response. Six rats were placed for 6 hours per day for 2 to 3 days (12 to 18 hours) in a low pressure chamber in which the pressure was maintained at 300 mm Hg (23,500 feet). On the day following the last chamber exposure, colchicine was administered. The mitotic percentage ranged from 5.6 to 8.5% with an average of 7.0%. The mitotic percentage values of the bled rats and of those subjected to low barometric pressure were significantly different from those of normal rats as determined by Fisher's "t" test. These results are presented in Fig. 3.

Discussion. The increased erythroid mitotic percentages found in the marrows of rats after

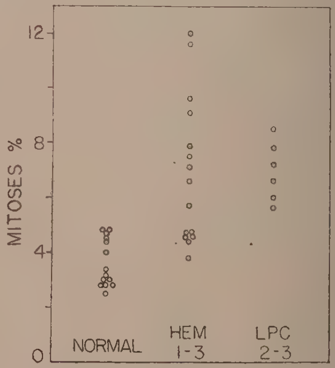

FIG. 3. Comparison of erythroid mitotic percentages of normal rats, rats 1 to 3 days after hemorrhage (HEM.) and those subjected to 300 mm Hg barometric pressure (LPC) intermittently 2 to 3 days (12 to 18 hr).

anoxic anoxia or hemorrhage indicate that physiological activity is being measured by the use of colchicine blockade. Increased numbers of mitoses were evident before a real increase in either the hematocrit or reticulocyte percentage was detected in the circulating blood.

The ability of the bone marrow to produce erythrocytes depends on the size and activity of the *functional* marrow volume. The total volume of erythroid marrow cells may be estimated by histological examination. Determination of the rate of mitotic division by the colchicine technic provides an empirical measure of the functional activity of the marrow.

The mechanism by which colchicine arrests mitotic division is unknown. It is not believed that the number of mitotic figures is increased by the alkaloid. Brues(7) found no evidence of stimulation of mitosis by colchicine in regenerating rat liver nor did Mellgren (8) in the developing adrenal gland.

The work reported involved the sacrifice of the rat in order to obtain adequate marrow samples. Preliminary observations indicate that in animals as large as the rabbit, marrow samples obtained by the needle puncture following colchicine administration give essentially the same results as those observed in the rat. Such a modification provides a technic capable of wider application.

Summary. 1. The influence of colchicine on arrest of erythroid mitoses of intact rat marrow was measured quantitatively. 2. A comparison was made after colchicine injection of the erythroid mitotic percentage of normal rats and of those of erythropoietically stimulated animals. Fourteen normal rats showed a range of 2.5 to 4.8%, average 3.6%. Fifteen rats, 1 to 3 days after moderate hemorrhage, gave 3.8 to 12.0%, average 6.9%. Six rats exposed for 6 hours per day for 2 to 3 days (12 to 18 hours) to a barometric pressure of 300 mm Hg yielded 5.6 to 8.5%, average 7.0%. Mitotic percentage increase above normal was evident in the stimulated animals before an established reticulocyte response was observed.

The author wishes to thank Professor Wilfred M. Copenhaver of the Department of Anatomy for his guidance in the identification of mitotic cell types and Mr. John Hegmann for his technical assistance.

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Received June 15, 1951. P.S.E.B.M., 1951, v77.

Effect of Therapeutic Doses of Ephedrine on Renal Clearances in Normal Man.* (18841)

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(Introduced by H. W. Smith.)

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On the basis of unpublished observations of Chasis, Goldring, and Smith(1), Smith states that ephedrine has no or a negligible effect on the renal circulation; he reproduces only one experiment in which 35 mg of ephedrine injected intravenously caused a small and probably insignificant decrease in renal plasma flow (diodrast clearance) and filtration rate (inulin clearance), and concludes that the mode of action of adrenaline and

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* This work was aided by a grant from The Commonwealth Fund.

[†] Present address, Department of Medicine, New York Hospital, Cornell University Medical School, New York.

[‡] Fulbright Fellow from the Philippines.

[§] Life Insurance Medical Research Fund Student Fellow.

1. Smith, H. W., *Lectures on the Kidney,* University Extension Division, University of Kansas, Lawrence, Kansas, 1943.

TABLE I. Effects of Therapeutic Doses of Ephedrine on Renal Hemodynamics in Man.

			Control values*			After ephedrine†			Change	
Subject	Dose, mg	Length of exp, min	Filtration rate, cc/min	Renal plasma flow, cc/min	Filtration fraction	Filtration rate, cc/min	Renal plasma flow, cc/min	Filtration fraction	Filtration rate, %	Renal plasma flow, %
Mc.‡	50	138	110	530	.21	109	498	.22	0	—6
V.	35	110	65	383	.17	70	399	.17	+ 7	+ 4
H.	50	119	149	580	.26	143	653	.22	— 4	+12
P.	75	110	148	797	.19	146	833	.18	— 1	+ 4
E.	50	180	111	695	.16	104	618	.17	— 6	—11
S.	50	139	85	542	.16	81	513	.16	— 4	— 5
W.§	75	205	140	580	.24	161	651	.25	+15	+12
K.‖	50	168	84	435	.19	81	415	.20	— 4	— 4

* Avg of 3 control periods.

† Avg of 4 to 8 periods.

‡ A brief period of syncope with hypotension accompanied by reduction in clearances occurred immediately after injection of ephedrine. This period was excluded.

§ $E_{PAH} = 94\%$. Renal A-V oxygen difference = 1.2 cc/100 cc. Both values remained constant.

‖ Ephedrine administered intramusc. except for K., in whom it was administered intravenously.

ephedrine is quite different.[¶] Subsequently, Ranges and Bradley(2), in a hemodynamic study, reported that 50 to 75 mg of ephedrine administered intramuscularly caused a significant decrease in renal plasma flow (diodrast clearance) with no change in filtration rate (mannitol clearance). They drew their conclusions from observations on 6 subjects but present no data. They postulate that adrenaline and ephedrine act in a similar manner. The earlier report of Smith, with its contradictory results, is not mentioned.

In view of the confusion on this matter, and because the importance of ephedrine as an agent for sustaining blood pressure in spinal anesthesia, etc., we have reinvestigated the renal response in normal subjects to therapeutic doses of this drug.

Methods. The subjects were 8 male convalescent patients from the wards of the Third Medical (New York University) Division of Bellevue Hospital without known cardiovascular or renal disease. The renal plasma flow was determined by the p-aminohippuric acid clearance (C_{PAH}) and the filtration rate by the inulin clearance (C_{IN}). Inulin was determined by Harrison's diphenylamine method as described by Goldring and Chasis(3), and PAH by the method of Smith, Finkelstein, Aliminosa, Crawford, and Graber(4). Pulse rate and blood pressure were measured every 10 minutes or oftener and the mean blood pressure calculated as one-third of the pulse pressure plus the auscultatory diastolic pressure, or measured directly by means of a calibrated mercury manometer connected to the brachial artery. After 3 control periods of 12 to 30 minutes each, 35 to 75 mg of ephedrine were injected intramuscularly and the overlying muscle massaged for 2 minutes; 35 mg were given intravenously to one subject. Clearance studies were continued for varying periods of time. The extraction ratio of PAH (E_{PAH}) and renal arterial-venous oxygen difference were measured in one subject at the mid-point of each clearance period by catheterization of the renal vein. Oxygen content was determined in duplicate by the method of Van Slyke and Neill(5).

[¶] The records of this laboratory for 1938 show 7 experiments, 2 of which were complicated by pyrexial hyperemia. In the other 5, ephedrine was administered intrav. or intramusc. in doses of 30 and 45 mg, respectively. The diodrast and inulin clearances were unaffected in all.

2. Ranges, H. A., and Bradley, S. E., *J. Clin. Invest.*, 1943, v22, 687.

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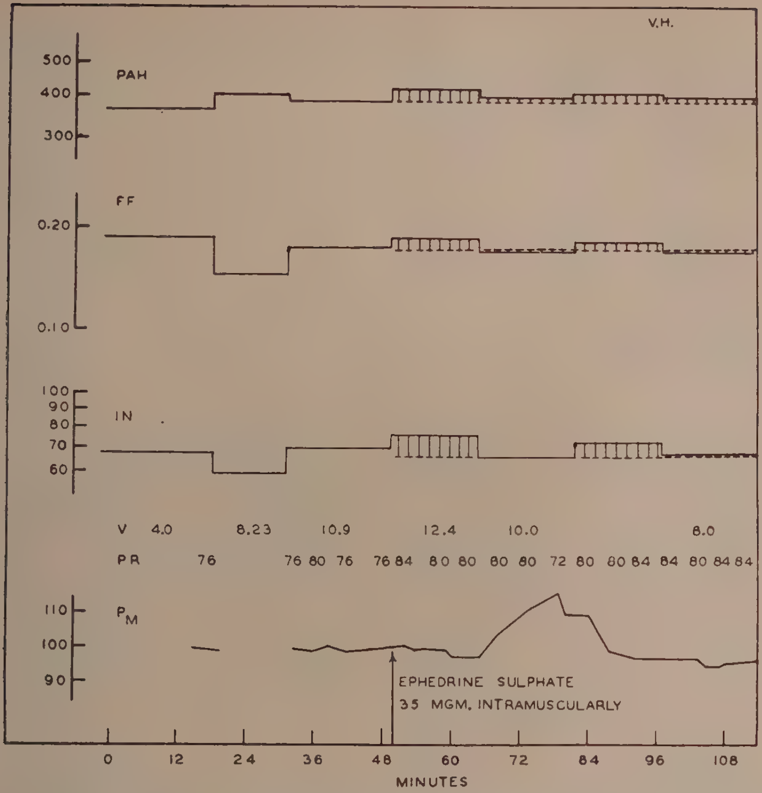

FIG. 1. Action of ephedrine on renal plasma flow (PAH or p-aminohippurate clearance in cc/min), filtration rate (IN or inulin clearance in cc/min), filtration fraction (FF or inulin/PAH clearance ratio), mean blood pressure (P_m in mm Hg), pulse rate (PR in beats/min) and urine flow (V in cc/min) in a normal subject.

Results. The results are tabulated in Table I, with a typical experiment shown in Fig. 1. In one instance we observed an 11% reduction in renal plasma flow and a 6% reduction in filtration rate, but clearance changes were negligible in the other 7 subjects.

It is generally agreed that in respect to cardiodynamic effects, ephedrine resembles adrenaline(2) although its action is more irregular(6). In these experiments, ephedrine regularly caused a small increase in systolic and diastolic blood pressure. The blood pressure generally began to increase within a few minutes after the administration of the drug, reached its maximum value in about 15 minutes (range 5 to 34 minutes), remained at this level for approximately 15 to 30 minutes and then slowly declined towards the control value. The increase in mean pressure on one occasion exceeded 40 mm Hg, and seemed unrelated to the dose of ephedrine (Table II). The pulse rate also increased slightly, usually by less than 10 beats per minute, and often remained elevated for the duration of the experiment.

Because the average values of several clearance periods, as shown in Table I, could mask transitory changes in renal hemodynamics, the clearances obtained during the period of maximal ephedrine effect, as judged by blood pressure and pulse rate, are shown in

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6. Starr, I., Gamble, C. J., Margolies, A., Donal, J. S., Jr., Joseph, N., and Eagle, E., *J. Clin. Invest.*, 1937, v16, 799.

TABLE II. Renal Clearances During Maximal Ephedrine Effect.

	Control values*		Period of maximal ephedrine effect†			
Subject	Filtration rate, cc/min	Renal plasma flow, cc/min	Filtration rate, cc/min	Renal plasma flow, cc/min	Change in mean blood pressure, mm Hg	Change in pulse rate per min
Mc.	110	530	114	542	+22	+12
V.	65	383	65	390	+10	+ 5
H.	149	580	144	620	+ 8	+ 4
P.	148	797	155	773	+14	0
E.	111	695	97	580	+26	0
S.	85	542	93	539	+16	+16
W.	140	580	127	616	+43	+ 9
K.	84	435	83	410	+21	+ 2

* Avg of 3 control periods.

† Value for single period of max ephedrine effect, as judged by increase in blood pressure and pulse rate.

Table II. Even these maximal effects show no consistent increase or decrease in renal plasma flow or filtration rate.

E_{PAH} and renal arterial-venous oxygen difference, measured in one subject (W.I.), remained constant at normal values of 94% and 1.2 cc/100 cc, respectively, throughout the course of the experiment, demonstrating that ephedrine does not inhibit tubular excretion of PAH or cause diversion of renal blood through arterial-venous channels.

Summary. Ephedrine in therapeutic doses (35 to 75 mg) administered intramuscularly to 7 subjects and intravenously to one failed to cause any consistent or significant change in renal plasma flow, filtration rate, PAH extraction ratio, or renal arterial-venous oxygen difference, despite an increase in mean blood pressure and pulse rate. These results are in agreement with the conclusion of Chasis, Goldring, and Smith(1) that, in marked contrast to adrenaline, ephedrine in therapeutic doses has no or a negligible effect on the renal circulation.

Received June 18, 1951. P.S.E.B.M., 1951, v77.

Potentiating Effect of Tetraethylammonium on Pressor Response to Epinephrine and Nor-Epinephrine.* (18842)

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The property of tetraethylammonium (TEA) of potentiating the pressor response to epinephrine and other pressor agents has been attributed(1) to the elimination of homeostatic blood pressure reflexes by this ganglionic blocking agent. In this connection, it is important to discover whether or not TEA has any direct potentiating effect on the action of epinephrine and it was the purpose of this investigation to attempt to shed some light on this aspect of the problem.

Methods. Twelve cats, weighing 2.23-5.57 kg, were anesthetized with ether after premedication with atropine sulphate 1.0 mg/kg. Both vagus nerves were divided and the spinal cord was then pithed in 10 animals after cervical laminectomy; in the remaining 2 animals the probe was passed through the orbit. The preparation was then left for an hour, body temperature being maintained constant by means of a warming device. Carotid blood pressure was recorded on a kymograph by

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* This study was supported by a grant from Parke, Davis Co.

1. Moe, G. K., Rennick, B. R., Capo, L. R., and Marshall, M. R., *Am. J. Physiol.*, 1949, v157, 158

TABLE I. Effect of Tetraethylammonium Chloride (TEAC) on the Hypertensive Response to l-epinephrine and l-nor-epinephrine. Pressor responses in mm mercury; mean ± S. D.*

	Control group		Test group		Difference	Significance†
l-epinephrine	Before saline	59.2 ± 3.9	Before TEAC	58.8 ± 8.7	.4	P <.5
	After 2 ml saline	62.7 ± 13.1	After 5 mg/kg TEAC	87.8 ± 18.1	25.1	P <.01
	Difference	3.5		29		
	Significance†	.5>P>.4‡		P <.01		
l-nor-epinephrine	Before saline	56.9 ± 10.8	Before TEAC	56.5 ± 6.4	.4	P >.5
	After 2 ml saline	62.9 ± 10.8	After 5 mg/kg TEAC	73.2 ± 11.8	10.3	P <.01
	Difference	6		16.7		
	Significance†	.2>P>.1§		P <.01		

* Computed from 2-4 pressor responses in each of 6 cats.

† "P" calculated by the *t* test of unpaired variates(2).

‡ When calculated by the *t* test of paired variates using the mean of 2 to 4 responses before and after saline, .5>P>.4.

§ When calculated by the *t* test of paired variates using the mean of 2 to 4 responses before and after saline, P >.01.

means of a mercury manometer, and injections were made intravenously into the external jugular vein at 5-minute intervals. Epinephrine and nor-epinephrine were administered alternately in approximately equipressor amounts to produce submaximal response. The doses required were, l-epinephrine,† 0.5-4.0 γ/kg; l-nor-epinephrine, 0.15-3.0 γ/kg. These amounts were made up to a volume of approximately 0.25 ml and were washed in with 0.5 ml of 0.9% saline. The pressor responses to the first 4 to 6 injections increased progressively in size and thereafter became more constant. Then in the test group of 6 cats, 2 to 4 pressor responses to both epinephrine and nor-epinephrine were elicited before, and again after, the administration of tetraethylammonium chloride in a dose of 5.0 mg/kg. Since the responses tended to change with time, a parallel control series of 6 cats was run in exactly similar manner except in that 2.0 ml saline was given in lieu of TEA.

Results. Blood pressure is expressed in terms of mm of mercury and responses as the mean and standard deviation of the absolute rise. After pithing, the baseline blood pressure remained remarkably constant in every cat though there was considerable variation between animals (32-86 mm of mercury). There was a tendency for the level to fall slightly, about 5.0 mm mercury, in some experiments after TEA. Since the potentiation of responses to epinephrine and nor-epinephrine always was much greater than this slight fall of pressure, it indicated not only a greater absolute rise but also a higher peak blood pressure.

Marked augmentation of the pressor response to epinephrine occurred after the administration of TEA (Table I). That this is not due to an increase in sensitivity of the preparation with the passage of time, is seen in the significantly greater response to epinephrine after TEA than after saline. That the test and control groups are comparable is seen in the fact that there was no significant difference between the responses before TEA and those before saline.

Similarly the pressor effect of nor-epinephrine was enhanced by the prior administration of TEA as is shown by a comparison of the responses following TEA with those following saline (Table I).

TEA potentiated the pressor response to epinephrine to a greater extent than that to nor-epinephrine (Table II) for there was no difference between their responses before TEA whereas there was a statistically significant difference after TEA.

The dose of TEA which was employed,

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† Pure l-epinephrine supplied by Parke, Davis Co. Dosage of l-epinephrine and l-nor-epinephrine expressed as base.

2. Snedecor, G. W., *Statistical Methods Applied to Experiments in Agriculture and Biology*, 4th edition. The Iowa State College Press, Ames, Iowa, 1946.

TABLE II. Comparison of Responses to Epinephrine and Nor-epinephrine. Pressor responses in mm mercury; mean ± S. D.*

	Epinephrine	Nor-epinephrine	Difference	Significance†
Before TEAC	58.8 ± 8.7	56.5 ± 6.4	2.3	.4 > P > .3
After 5 mg/kg TEAC	87.8 ± 18.1	73.2 ± 11.8	14.6	P < .01

* Computed from 2-4 pressor responses in each of 6 cats.
† "P" calculated by the *t* test of unpaired variates(2).

5.0 mg/kg, was apparently nearly optimal in producing potentiation since the further administration of 10.0 mg/kg did not result in any additional potentiation of pressor responses in the test group when compared with the saline controls.

Discussion. From the results of this investigation one might conclude that TEA, in doses of 5.0 mg/kg intravenously in pithed cats, potentiated the pressor responses to both epinephrine and nor-epinephrine, there being a higher peak response in addition to a greater absolute rise of blood pressure. Since this was demonstrated in vagotomized animals with pithed spinal cords, such potentiation cannot have been due to the modification of cardiovascular reflexes.

Page and Taylor(3) reported the potentiation of the pressor action of epinephrine by tetraethylammonium in intact, anesthetized dogs. The mechanism they postulated was either an explosive manifestation of the sensitization of denervation or the blocking of amine oxidases. Their findings were confirmed subsequently in unanesthetized dogs (4) and by Moe(5). From simultaneous measurements of blood pressure and femoral arterial flow he concluded that the augmentation of pressor responses was due to the elimination of homeostatic cardiovascular reflexes. These are eliminated by virtue of the ganglionic blocking properties of TEA(6). This hypothesis, now supported by Page and Taylor(7), is strengthened by the fact that TEA potentiates the pressor response to other agents(1) and also enhances the fall of pressure produced by depressor substances(5), even that which follows the slow infusion of epinephrine(1). Moe also reported that, in intact anesthetized animals, the absolute rise of pressure in response to epinephrine was greater after TEA, but since the rise began from the hypotensive level established by the agent, the maximum was little greater than before. Stutzman *et al.*(8) report similar findings for phenylephrine after TEA.

The enhancement of epinephrine pressor effects by the blocking of compensatory reflexes, as is suggested by the evidence from the reports which have been cited, leaves unexplained however the potentiation of "Isuprel" (N-isopropyl homologues of epinephrine) tachycardia by TEA which at the same time augments the depressor response to the drug(4); nor does it explain the finding that hepatectomy abolished the augmenting action of TEA(3). There are few reports concerning the possibility of an additional direct mechanism although Moe(5) makes the statement, unsupported by published data, that, in the pithed cat, TEA does not appreciably alter the pressor action of epinephrine. This is at variance with the findings herein shown which would indicate that TEA does possess the property of potentiating the pressor actions of epinephrine and of nor-epinephrine independently of the central nervous system.

A peripheral effect of TEA in modifying responsiveness to various substances has been demonstrated by Collins(9). Using isolated intestine he reported augmentation of re-

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3. Page, I. H. and Taylor, R. D., *J.A.M.A.*, 1947, v135, 348.

4. Corcoran, A. C. and Page, I. H., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 148.

5. Moe, G. K., *J.A.M.A.*, 1948, v137, 1115.

6. Acheson, G. H. and Moe, G. K., *J. Pharm. and Exp. Therap.*, 1946, v87, 220.

7. Page, I. H. and Taylor, R. D., *Circulation*, 1950, v1, 1233.

8. Stutzman, J. W., Pettinga, F. L., Fruggiero, E. J. and Maison, G. L., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 686.

9. Collins, D. A., *J. Pharmacol. Exp. Therap.*, 1948, v94, 244.

sponses to angiotonin and histamine and variable results with barium chloride and acetylcholine. Stone(10) found that TEA potentiated the motor effect of epinephrine on the seminal vesicle of the guinea pig. Finally, suggestive evidence was presented by Stutzman *et al.*(8), who reported that epinephrine-induced ventricular tachycardia was enhanced by tetraethylammonium chloride, even in spite of effective sympathetic block, and supradiaphragmatic splanchnicectomy.

Summary. 1. The potentiation by TEA of the pressor responses to epinephrine and nor-epinephrine has been demonstrated in cats with spinal cord pithed. 2. The effect of epinephrine was enhanced to a greater extent than that of nor-epinephrine. 3. This potentiation was not due to the modification of homeostatic cardiovascular reflexes.

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10. Stone, C. A., 1951, unpublished data.

Received June 18, 1951. P.S.E.B.M., 1951, v77.

Effects of a Pancreatic Preparation (Viokase) in Depancreatized Cats. (18843)

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Substitution therapy for patients or animals which are deficient in the external secretion of the pancreas is still unsatisfactory. The various pancreatic preparations have afforded only limited replacement. Depancreatized carnivorous animals have usually been fed raw pancreas for its digestive and lipotropic effect. Even this falls far short of the normal external secretion. Recently a desiccated, defatted pancreatic powder (Viokase)† has been tried in depancreatized cats in this laboratory. The effect of this powder on the mass and fat content of the stools has been examined. Its influence on the insulin requirement has been observed because of Machella's unpublished report that the insulin requirement of a depancreatized man was increased during its administration.

Methods. Cats were depancreatized under nembutal anesthesia and the completeness of the operation confirmed by the amount of glucose excreted during fasting on the first one or 2 post-operative days. After this, a few days on insulin and penicillin put the animals in excellent condition. They were kept in metabolism cages. The daily excretion of glucose was determined and the dose of insulin adjusted accordingly. The stools of each cat were collected daily before the cages were cleaned, and were pooled for each metabolic period; the pooled samples were then dried to constant weight in a hood and analyzed for total fat by the method of Fowweather(1) adapted for use on the dried stool. Duplicate or triplicate analyses were performed on each sample.

The diet for all animals during all periods used for fat analyses (Tables I and II) was 150 g of raw horse meat daily. The studies on insulin requirement included some animals on 150 and some on 200 g of meat. The meat was quite constant in its composition. Analyses of different lots showed that it contained 73% water; 27% solids (dry weight); 20.8 g of protein per 100 g meat based on the determination of nitrogen. This means that the 150 g diet contained 31 g of protein, and 41 g dry weight. Two fat analyses showed 0.082 and 0.093 g of fat per gram dry weight of food. Based on the average of these measurements, the daily fat intake was 3.57 g per day. During the periods on Viokase, 2 level teaspoonfuls (about 3 g) of this powdered pancreas was mixed with the meat. Because of its small

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* National Institutes of Health Postdoctorate Research Fellow, 1949-50.

† Manufactured by the Viobin Corp., Monticello, Ill.

1. Fowweather, F. S., *Brit. J. Exp. Path.*, 1926, v7, 7.

TABLE I. Wt and Fat Content of Feces of Cats Under Various Conditions (Diet Constant).

Condition	Cat No.	Period, days	Fecal wt: Wet, g per day	Fecal wt: Dry, g per day	Fecal fat excretion: Conc.,* mg/g dry wt	Fecal fat excretion: Amt, g/day	Fecal fat excretion: % of ingested fat
Normal	M-9	9	3.5	—	—	.17†	5
	M-11	7	2	1.03	146	.15	4
	M-15	5	3	1.16	173	.20	6
	M-16	4	2.9	2.48	164	.41	12
Depancreatized receiving Viokase	M-7	8	4.3	—	—	1.73†	48
	M-7	7	10.2	4.62	309	1.43	40
	M-1	8	13.6	8.06	478	3.87	''100''
	M-14	6	13.2	9.25	495	4.58	''100''
	M-18	4	9.4	6.33	625	3.89	''100''
Depancreatized without Viokase	M-14	4	57	24.20	323	7.83	''100''
	M-23	4	24.5	8.38	234	1.96	55
	4	4	26	11.76	235	2.75	77
	8	5	42	15.35	231	3.54	99

* Avg of duplicate determinations on pooled sample of period.
† Based on analysis of fresh stool.

TABLE II. Avg Values of Fecal Wt and Fat Excretion (Diet Constant).

Condition	Fecal wt: Wet, g per day	Fecal wt: Dry, g per day	Fat excreted: Conc., mg/g dry wt	Fat excreted: Amt, g/day
Normal	2.9	1.55	161	.22
Depancreatized; on Viokase	10.7	7.24	464	3.23
Depancreatized; without Viokase	37.4	14.92	256	4.02

amount and freedom from fat this has been disregarded in the calculation of results.

Results. Table I presents the observations with and without the addition of Viokase to the constant meat diet. In both groups of depancreatized animals a marked variation in the weight and fat content of the stools was observed. The increased total and solid weight of the stools after pancreatectomy followed the pattern described in dogs by Selle and Moody(2). The most striking effect of Viokase is the reduction in the bulk and dry weight of the stools to half that of the untreated depancreatized series of animals. Table II summarizes the averages which because of their small number and wide range do not merit statistical analysis. However, the effect of Viokase on the weight of the stool is regarded as significant.

When the data on fat excretion are examined, it is seen that the concentration of fat tended to be greater in the treated series. However, the amount of fat excreted is essentially the same for both groups of depancreatized cats. This resembles the results obtained in dogs treated with pancreatin(2). The last column of Table I, which expresses the fat excreted as per cent of the ingested fat permits comparison with the results in depancreatized dogs studied by Coffey, Mann, and Bollman(3). They found that fat excretion amounted to 54 to 76% of the dietary fat. Our cats which excreted 40 to more than 100% indicate the range and irregularity of this finding.

During the administration of Viokase, the bulk of the stools was decreased without apparent alteration in fat absorption. In Cat M-14, which was studied on both regimens (Table I), the fecal nitrogen was determined. When given Viokase, this amounted to 0.4 g of nitrogen daily. Without Viokase the fecal nitrogen was 1.9 g per day. These values represent 2.5 and 12 g of protein respectively. Others have described the impaired protein absorption after pancreatectomy and its partial prevention with pancreatin(3). The single observation on Cat M-14 is in accord with these results.

The insulin requirement. In the studies

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2. Selle, W. A., and Moody, I. W., *J. Nutrition*, 1937, v13, 15.

3. Coffey, R. J., Mann, F. C., and Bollman, J. L., *Am. J. Digest. Dis.*, 1940, v7, 141, 144.

TABLE III. Insulin Requirement of Depancreatized Cats.

		Avg values			
Group*	Dietary supplement	Meat per day, g	Insulin/day, units	Glycosuria/day, g	Initial wt, kg
A	Raw pancreas	182	9.5	3	2.3
B	Viokase	180	15.4	8.7	2.7

* A—based on 6 metabolic periods of 3 to 5 days each in 5 cats; B—based on 7 metabolic periods in 4 cats.

cited(2,3) no mention is made of the effect of any replacement treatment on the insulin requirement of depancreatized dogs. One would expect that improved digestion might influence the need for insulin and the data in Table III suggests that this is so. In Table III, group A consists of animals prepared 10 years ago when raw pancreas (50 g daily) was part of the diet. Group B is composed of animals on Viokase, some of which were not studied for fat excretion and received diets of 200 g. The average diet and body weight for each group is similar. The cats given Viokase received almost twice the amount of insulin used in group A, but in spite of this excreted nearly 3 times as much glucose in the urine. The dose of insulin varied widely in different cats (6 to 24 units per cat in the past few years in this laboratory). For this reason one must be guarded in the interpretation of such averages. Nevertheless, the number of animals and of metabolic periods suggest that there is a true and possibly striking effect of Viokase on the insulin requirement. When one surveys the reports on impaired pancreatic digestion and the relative inadequacy of available substitutes, it is not surprising that the depancreatized animal(4) and man(5) require less insulin than their counterparts (pituitary or alloxan diabetes(6,4) and clinical diabetes) in which an essentially normal external secretion is present.

The general usefulness of Viokase in depancreatized cats may be summarized. It is more convenient, and probably cheaper, to add a stable powder to the diet than to procure fresh pancreas. The steatorrhea and loss of body weight which follow pancreatectomy are perhaps better controlled than with raw pancreas. In general, Viokase has kept depancreatized cats in satisfactory condition. The effect on insulin requirement, which may be regarded as an index of improved digestion, suggests that this pancreatic preparation deserves clinical trial, especially when faulty absorption of protein is present.

Summary. A pancreatic preparation (Viokase) has been used to substitute for the external secretion in a series of depancreatized cats. It had no effect on fat absorption but diminished the bulk of the stools, probably by improving the digestion of protein. The improved digestion was accompanied by an increased requirement for insulin.

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4. Thorogood, E., and Zimmerman, B., *Endocrinology*, 1945, v37, 191.

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Received June 20, 1951. P.S.E.B.M., 1951, v77.

Detoxification of Alloxan by Borate.* (18844)

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It has recently been reported(1) that normal subjects excrete an alloxanic acid-like compound which on hydrolysis yields oxo-

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* Supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

† Present address: Army Medical Service Graduate School, Army Medical Center, Washington, D. C.

1. Seligson, D., Seligson, H., Shapiro, B., Paley, R. G., Riaboff, T. and Lukens, F. D. W., *Fed. Proc.*, 1951, v10, 124.

malonic acid. This in turn suggests that alloxan may be an intermediate metabolic product in man. If calculated as alloxan, the oxomalonic acid which was isolated would represent a urinary excretion of about 100 mg per day. If urinary oxomalonate arises from alloxan, which is a likely possibility, then it is desirable to learn more of the biochemical reactions of alloxan. It is the purpose of this report to show that borate detoxifies alloxan in a hitherto unrecognized manner. Rose and Gyorgy(2) have reported that boric acid injected into rats prior to alloxan prevents the development of alloxan diabetes, but they did not study the chemical reactions involved.

Seligson and Seligson(3) found that alloxan in neutral buffers is converted to alloxanic acid and that the reaction was catalyzed by hydroxyl ions. The conversion was a rapid first order reaction with alloxan being half decomposed in 2 to 4 minutes depending upon temperature and pH (Fig. 1, curve O). Alloxan in plasma behaved similarly, half the alloxan having disappeared in 5 minutes (Fig. 2, curve O).

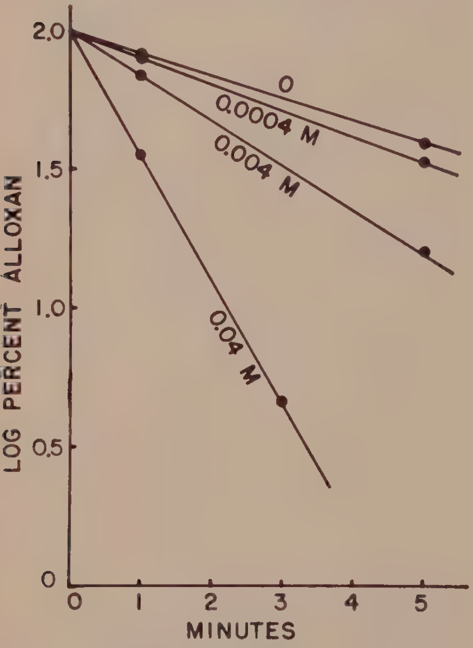

FIG. 1. Curves represent the log of the per cent of added alloxan remaining in a 0.1 M phosphate reaction mixture during the course of the reaction. Figures adjacent to each curve show the concentration of added borate in the reaction mixture. The pH at 5 min was 7.1 for each mixture.

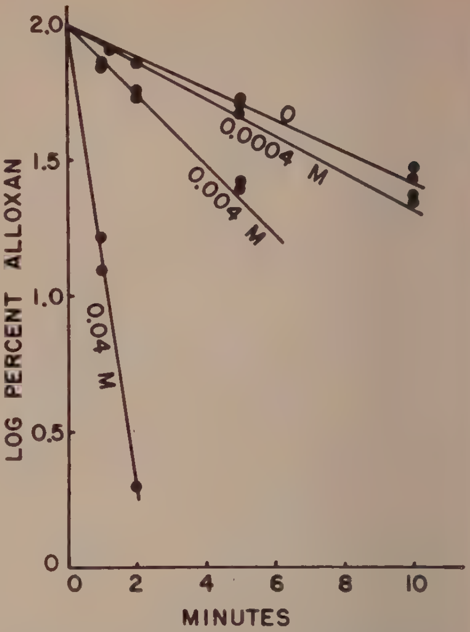

FIG. 2. Curves represent the log of the per cent of added alloxan remaining in a reaction mixture containing plasma and varying amounts of borate. Reaction mixtures at 5 min were pH 7.4.

Alloxanic acid contains a free carboxyl group which is readily and stoichiometrically oxidized by ceric sulfate yielding parabanic acid and 1 molecule of carbon dioxide. Alloxan is not so oxidized. Thus, measurement of the ceric sulfate consumed or CO_2 liberated provided a method for following the kinetics of the conversion of alloxan to alloxanic acid. A microdiffusion method(4) was used for measuring the liberated carbon dioxide.

Procedure. Phosphate buffer‡ 5 parts water 4 parts and alloxan‡ 1 part, were rapidly mixed

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2. Rose, C. S., and Gyorgy, P., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 746.

3. Seligson, D., and Seligson, H., *J. Biol. Chem.*, 1951, v190, 647.

4. Seligson, D. and Seligson, H., in preparation.

‡ The phosphate buffer solution was 0.2 M and its pH was 7.49. The alloxan solution was 0.01 M in 0.001 N HCl.

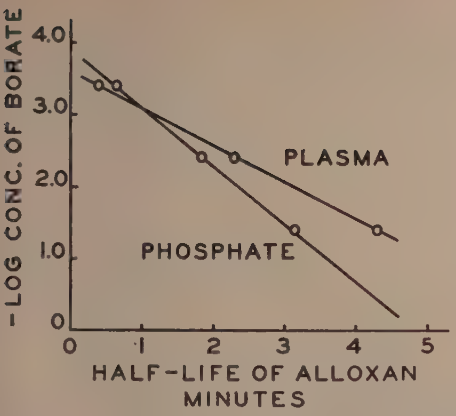


FIG. 3. Log of the concentration of borate plotted against the half life of alloxan added to a phosphate buffer or plasma follows a straight line function.

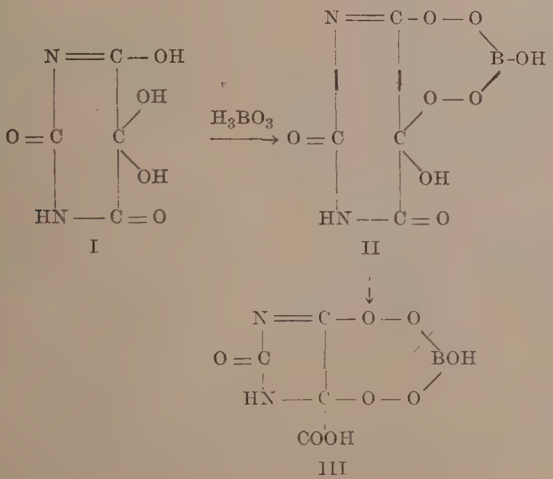
and periodically 2 ml of reaction mixture was rapidly transferred to 3 ml of 6 N H_2SO_4. 0.5 ml was treated with 1 drop of ceric sulfate reagent (ceric sulfate, 0.3 N in 3 N H_2SO_4 containing sufficient osmium tetroxide to make the solution 0.00001 M) and the liberated carbon dioxide was measured by a microdiffusion procedure(4). A reagent blank was run for the CO_2 content of the system, using water instead of alloxan. In this manner the values for alloxan, shown in Fig. 1, were obtained. The effect of borate was measured by substituting dilutions of the borate buffer (0.1 M and pH 7.40) for the 4 ml of water above and thus making reaction mixtures 0.0004 M, 0.004 M, and 0.04 M with respect to borate.

Plasma was studied in the above systems by replacing the 5.00 ml of phosphate buffer with plasma diluted 1:2.

Results. The data shown in Fig. 1 and 2 show the remarkable accelerating effect of borate on the conversion of alloxan to alloxanic acid. Increasing concentrations of borate accelerate the reaction in a regular manner as shown in Fig. 3, where the log of the concentrations of borate plotted against the half life of alloxan gives straight lines in phosphate buffer and in plasma.

Discussion. The effect of borate in catalyzing the reaction may be through a Boeseken type ester as follows: At pH 7.4 alloxan forms an enol (I) which results in a cis-dehydroxy form and this is assumed to form a Boeseken type of ester (II) which quickly goes to the more stable alloxanic acid borate complex (III). Evidence for the latter is obtained by the greater difficulty in oxidizing alloxanic acid in the presence of borate and also the greater acidity of the borate alloxanate complex.

An explanation on the protective effect of boric acid(2) against the diabetogenic effect of alloxan in rats is supplied by the accelerating effect of borate on the conversion of toxic alloxan to non-toxic alloxanic acid(5). This



constitutes a previously unrecognized mechanism for the destruction of alloxan differing from those based on reduction as by glutathione(5), or condensation(5).

Conclusion. Borate greatly accelerates the conversion of alloxan to alloxanic acid. This observation provides an explanation for its protective effect against alloxan diabetes.

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Received June 20, 1951. P.S.E.B.M., 1951, v77.

Use of Desoxycorticosterone Acetate in Treatment of "Hyperheparinemia".* (18845)

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It has long been assumed that mast cells contain heparin in an active or inactive form(1). Recently, evidence has been presented(2) to show that, in response to certain hormonal products, chiefly of the stress-producing type, the morphologic pattern of the mast cells of mice and other animals are altered and, in the animals studied, the heparin content of the blood may increase. Of the hormones studied, only desoxycorticosterone acetate (DCA) was found to preserve the morphology intact and, in larger doses, prevented its alteration by subsequent administration of the stress-producing hormones(2).

Recently, an opportunity was afforded to study a case of idiopathic "hyperheparinemia"(3). Since this report, we have studied the effect of protamine sulphate and DCA on this patient's coagulation defect. The response to protamine therapy was excellent but, after a month, evidence of resistance to protamine appeared and he relapsed. Because of his subsequent failure to respond to protamine sulphate, it was decided to try the effect of large doses of DCA on this patient. The administration of 25 mg of DCA in oil daily by the intramuscular route resulted in a drop of the endogenous heparin from 23 γ per ml of plasma to 0 γ in 4 days (Table I).

During the course of observation, this patient, under control conditions, showed a clotting time (Lee-White) of 110-130 minutes, a prolonged one stage prothrombin time (Quick) of 75-90 seconds (normal 13.2 seconds), despite a normal prothrombin concentration by the modified 2-stage procedure of Ware and Seegers(4), and a prolonged recalcification time of oxalated plasma. Both the protamine titration test(5) and the protamine-heparin titration method(6) gave consistently abnormal values under control conditions. During therapy with DCA, the three other abnormal tests improved along with the drop in the protamine titration. A complete reversion to normal values occurred when the endogenous heparin had fallen to an insignificant amount in the plasma.

To rule out the possibility of a spontaneous remission, treatment was discontinued for 72 hours following which hyperheparinemia recurred. On the readministration of 90 mg of DCA in a period of 4 days, the heparin level of the blood again fell from 23 to 2 γ per ml of plasma. This observation suggests the possible use of this therapy in the hemorrhagic

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* Aided in part by a grant from Eli Lilly and Co., Indianapolis.

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2. Stuart, G. E., *Anat. Rec.*, 1951, v109, 351.

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4. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

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TABLE I.

Day of treatment	Clotting time (min)	1-stage prothrombin (sec)	Recalcification time of oxalated plasma (sec)	Endogenous heparin in plasma (γ/ml)
Before DCA	137	142	270	23
After 2 days	131			23
3 ”	72			17
4 ”	14	13.8	90	0

tendency following irradiation(5).

In this patient, the use of desoxycorticosterone acetate, in large doses, reduced the level of heparin or heparinoid substances in the blood. The mechanism of this response is unknown.

Received June 20, 1951. P.S.E.B.M., 1951, v77.

Characteristic Electrophoretic Patterns in Hemophilia and Idiopathic Thrombocytopenia.* (18846)

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Observations on the changes in the composition of plasma proteins in the hemorrhagic diseases as revealed by electrophoresis are practically non-existent. Armstrong(1) failed to find any fundamental electrophoretic difference between normal and hemophilic plasma. No data are available in the literature on the electrophoretic patterns in idiopathic thrombocytopenia.

Routine electrophoretic analyses of plasma, by means of the technic described in a previous communication(2), have been conducted during the past year in a group of cases with various types of hemorrhagic manifestations. This group included 5 cases of hemophilia, 8 of idiopathic thrombocytopenic purpura, 6 of thrombocytopenia secondary to hypoplastic anemia or leukemia, 3 with the "anaphylactoid" type of vascular purpura, and 6 with vascular purpura. A characteristic anomaly of the electrophoretic patterns was observed in 17 of these 28 patients. The anomaly consisted in: (a) the appearance of an unusual peak in the group of the α-globulins, which we have designated as α_x (Fig. 1), with a motility ranging between 4.30 and 4.60 $\times$ 10^{-5} cm^2 volt^{-1} sec^{-1} (in veronal-citrate buffer, pH 8.6, ionic strength 0.1); (b) complete disappearance of the usual α_2- and α_3-globulin peaks with mobility between 3.75 and 4.25 $\times$ 10^{-5} cm^2 volt^{-1} sec^{-1}. These changes were more marked in the ascending than in the descending boundaries.

These anomalies were observed in all the cases of hemophilia and idiopathic thrombocytopenia which were studied; they were also found in 2 of the 6 cases of secondary thrombocytopenia, in 2 of 3 cases of the "anaphylactoid" type of vascular purpura. The characteristic anomaly was also observed in 2 cases of acquired hemolytic anemia, one of which

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* This study was supported by grants from the American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

† This work was completed during the tenure of a Damon Runyon Senior Clinical Research Fellowship, administered by the American Cancer Society.

1. Armstrong, S. H., Jr., Quoted by Lewis, J. H., *et al., J. Clin. Inv.*, 1946, v25, 870.

2. Bernfeld, P., Bonner, C. D., and Homburger, F., *Proc. Second Clin. ACTH Conf., Blakiston Co.*, 1951, v1, 282.

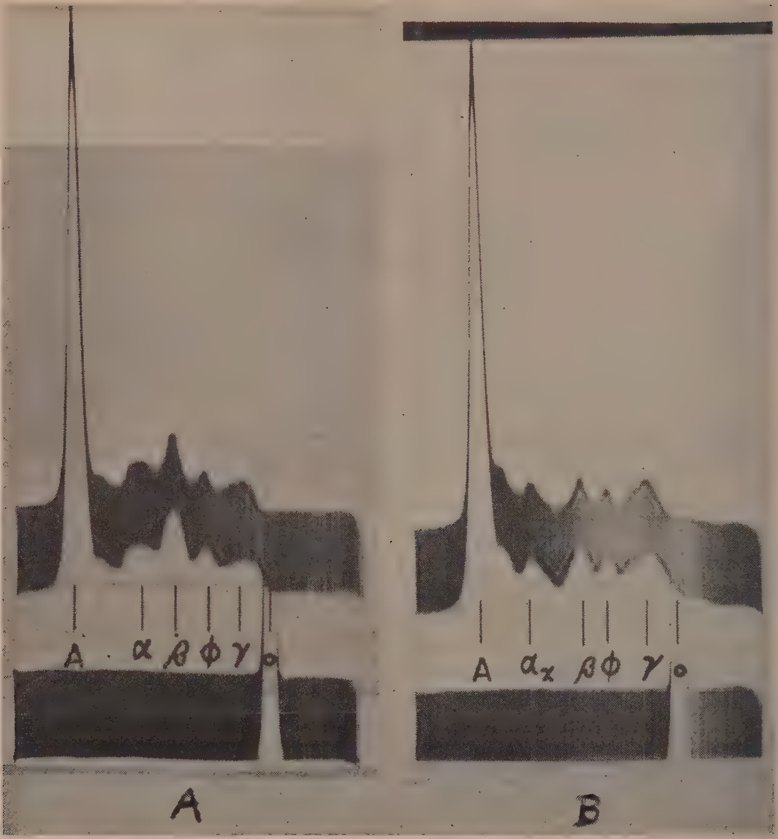

FIG. 1. Characteristic electrophoretic patterns in plasma of patients with hemophilia or idiopathic thrombocytopenic purpura. (A: normal plasma; B: plasma with characteristic anomaly).

presented thrombocytopenia (Case M.G.), and the other one evidence of platelet dysfunction as demonstrated by an elevated prothrombin activity of serum (Case O.K.). On the other hand, the described abnormalities were not evident in numerous other cases (164) studied in our laboratories. These included healthy individuals and patients with a large variety of pathologic conditions. A few of these control cases exhibited a peak with mobility similar to that of the α_x but both α_2- and α_3-globulin peaks were present in normal amounts. Pertinent laboratory findings of all the cases with the characteristic electrophoretic anomaly are collected in Table I.

The interpretation of the findings presented in this paper is difficult, since the characteristic patterns described were found in a variety of hemorrhagic diatheses, which are apparently unrelated in their pathogenetic mechanism. From the technical point of view, it cannot be stated with certainty whether the modifications described here indicate the presence of a particular protein or a mere anomaly. Nevertheless, it appears significant that such characteristic electrophoretic patterns were consistently found in hemophilia and idiopathic thrombocytopenic purpura. In hemophilia, transfusion of fresh plasma (Case D.C.) and the injection of 200 mg of Corti-

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3. Ivy, A. C., Shapiro, P. F., and Melnick, P., *Surg., Gynec. and Obst.*, 1935, v60, 781.

TABLE I. Pertinent Laboratory Data in Patients Exhibiting the Characteristic Electrophoretic Anomaly. (A normal case is given as a control).

Name	Age	Sex	Clotting time (Silicone),* min	Bleeding time,† min	Platelets $(\times 10^{-3})$ per mm^3	Tourniquet test‡ petechiae	Clot retraction,§ %	Prothrombin activity of serum,‖ %	Fibrinogen, mg/100 ml	Serum accelerator activity, %
(A) Hemophilia										
D.C.	4	M	1340	5½	560	11	43	95/100	472.4	12
H.N.	39	M	1620	4	529.3	4	51	69/87	407	38
R.S.	43	M	1724	5½	464.8	7	44	89/94	364.7	29
R.So.	9	M	1587	5½	754.2	8	43	90/92	474.9	10
S.F.	1	M	1427	7	551.8	1	24	57/90	229.5	21
(B) Acute idiopathic thrombocytopenic purpura										
A.R.	42	F	48	18	53.4	19	4	57/90	279.4	35.5
H.F.	16	M	37	>20	26.5	18	15	62/95	471	28.5
T.M.	2	M	28	13	20.9	12	12	80/100	307.2	42
A.W.	15	M	49	>20	13	16	9	75/100	401.9	24.2
E.F.	16	F	32	19	8.2	27	0	89/100	297.2	29.5
H.C.	56	M	35	>20	24.5	15	12	21/41	175.4	27
R.A.	6	M	41	>20	11.1	24	5	40/100	375.8	13.6
V.D.	6	F	33	18	58.3	18	10	62/95	403.6	25.8
(C) Miscellaneous										
C.B.¶	16	F	29	18	30.9	44	7	75/95	179.4	14.5
J.T.¶	56	M	37	>20	23	65	2	60/80	371.4	27
M.R.**	5	F	28	5½	616.9	2	37	4.7/100	379.2	77
B.W.**	3	M	36	4	800.42	15	32	15/100	456.1	91
O.K.††	34	F	31	5½	424.5	4	21.5	74/79	368.5	59.5
M.G.††	44	F	40	6½	180	9	19.5	32/69	287.4	34
(D) Normal										
	33	M	37	2½	560.8	2	39.5	8/100	435.7	79.5

* Blood was collected with Silicone-coated syringes and needles and transferred to Silicone-coated test tubes.

† Method of Ivy(3).

‡ Expressed as number of petechiae in an area of 1 inch diameter on the anterior surface of the forearm, 2 fingers below the antecubital fossa, after 10 min of application of mean blood pressure.

§ Expressed as ml of serum obtained from 1 ml of whole blood (× 100).

‖ Figure at numerator indicates prothrombin activity of serum, figure at denominator gives prothrombin activity of plasma.

¶ Hypoplastic anemia. ** Anaphylactoid purpura. †† Hemolytic anemia.

sone intramuscularly (Case R.S.) failed to modify the described anomaly. This occurred equally in one patient who had never received transfusions previously (Case S.F.) and in 4 others to whom repeated plasma and blood transfusions had been administered. In the latter group no precipitating antibodies against human plasma or antihemophilic globulin could be demonstrated. In the idiopathic thrombocytopenia group, the characteristic electrophoretic patterns were found to be still present 6 months after recovery of the patients, following either spontaneous remission of the disease (Case R.A. and H.F.) or successful splenectomy (Case T.M. and E.F.). Further investigations are in progress.

Summary. Characteristic electrophoretic patterns have been observed in all 5 cases of hemophilia and in all 8 cases of idiopathic thrombocytopenic purpura. These patterns show the following characteristics: (a) appearance of an unusual peak in the group of the α-globulins (α_x), and (b) disappearance of the usual α_2- and α_3-globulin peaks. The same anomaly has been observed in some cases of secondary thrombocytopenia, "anaphylactoid" type of vascular purpura, and hemolytic anemia with evidence of platelet dysfunction.

The authors wish to acknowledge gratefully Dr. William Dameshek's helpful suggestions and comments during the above investigation.

Received June 22, 1951. P.S.E.B.M., 1951, v77.

Further Observations on Cold Hemagglutinin Activity in Encephalomyocarditis, M.M., Mengo Encephalomyelitis and Columbia-SK Immune Sera. (18847)

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Cold hemagglutinins are serum entities which possess the property of aggregating human erythrocytes, in particular erythrocytes of the O group, at low temperatures. Stats and Wasserman(1) have reviewed the subject quite extensively, and the introductory descriptive material below derives in the main from that source.

Cold hemagglutination was first recognized by Landsteiner(2) who showed it to depend upon a definite antigen-antibody reaction. The reaction can be distinguished from the usual iso- or hetroagglutination by its high sensitivity to thermal change; as a rule it occurs to only a slight extent or not at all above 22°C. The phenomenon of agglutination in the cold can be reversed by warming to temperatures above 20°C and caused to recur by cooling to below 10°C. Cold hemagglutinins do not lose their activity after heating to 56°C for 30 minutes, and, like other agglutinins, they can be stored in the cold with only slight diminution in potency. Cold hemagglutinins have been described(1) as gamma globulins. Antibody nitrogen studies have also revealed that a titer of cold agglutinins of 1:2560 is equivalent to 1.473 mg nitrogen per ml.

Positive cold hemagglutination reactions have thus far been demonstrated chiefly with sera from cases of primary atypical pneumonia. The only other sera which elicit this phenomenon with any regularity are those from individuals with African trypanosomiasis. Cold hemagglutinins at high titers have also been reported in sera from cases of acquired hemolytic anemia and in some patients with Raynaud's disease(1).

A preliminary serological study(3) showed an increased activity of cold agglutinins in the sera of rabbits immunized with a mouse brain suspension of encephalomyocarditis virus. This suggested that perhaps other viruses, particularly those of murine poliomyelitis, or as Warren *et al.*(4) suggest, the encephalomyocarditis group, might also induce this response. The experiments described below were carried out to determine the extent to which these viruses may elicit cold hemagglutinin production, and to investigate the possibilities of such a technic in providing an additional differential criterion for the encephalomyocarditis group.

Preparation of antisera. Ten per cent suspensions of infected mouse brain in isotonic saline were used as immunizing antigens. The rabbits employed were not of an inbred stock, but stemmed from several different families. They were 10 weeks of age or older at the start of the experiment; this in light of past studies by Wheeler(5) who found that the normal cold-agglutinin titer of newborn rabbits diminished considerably during the first weeks following birth. Two or 3 rabbits were used for each virus. Prior to immunization all rabbits were bled for normal specimens of serum. At weekly intervals, over a period of 3 weeks, 1 cc amounts of the antigen suspensions were injected into the animals by the intramuscular route. During the fourth and fifth weeks 2 cc and 3 cc amounts respectively, were introduced intraperitoneally. Following a 10-day rest period the animals were bled from the heart. The blood samples were al-

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* Grateful acknowledgement is made to Mr. Alfred Nell for his technical assistance.

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2. Landsteiner, K., *München. med. Wschuschr.*, 1903, v50, 1812.

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lowed to clot for 2 to 3 hours at room temperature, and the expressed sera were removed, spun, and then stored at —20°C until ready for testing. The titers of the inocula employed, expressed as the reciprocals of the logarithms of the LD_{50}, were 8.1, 8.5, 7.9 and 7.0 for EMC, M.M., Mengo, and Columbia-SK, respectively.

Cold hemagglutination test. Although all human erythrocytes can be agglutinated in the cold by hemagglutinating sera, group O cells were used because they were found generally to yield highest titers. The human O blood employed was stored in an equal volume of Alsever's solution at refrigerator temperature; prior to testing the cells were washed 3 times in isotonic saline and then brought up to 2% by volume with saline.

Progressive 2-fold dilutions of serum in saline, from undiluted to 1:512, were pipetted in 0.05 cc quantities into serological tubes (10 x 75 mm), and 0.05 cc of the 2% red cell suspension was added to each tube. The tubes were shaken and then placed in the refrigerator (4°C) for 30 minutes. At the end of this time the tube mixtures were centrifuged for 1 minute at 1500 rpm and returned to the refrigerator for another 30 minutes, following which they were read for agglutination under the low power of the microscope. To gain some idea about the thermal range of the reaction for the several sera, similar tests were carried out at 22°C and at 37°C. In addition, reversals of the reaction were studied, those sera initially tested at 4°C being later studied at 37°C and those tested at 22°C and 37°C later studied at 4°C. All readings were carried out rapidly at room temperature, the end point being determined by the last tube showing distinct clumping under low power magnification.

Results. The findings are condensed in Tables I and II. The former delineates the results in terms of average titers, while the latter pictures the outcome of tests with pooled sera. Titers, in both cases, are expressed as the reciprocal of the highest dilution of serum which gave agglutination.

TABLE I. Cold Hemagglutinins in Rabbit Sera for Human O Erythrocytes both Before and After Immunization with Viruses of the Encephalomyocarditis Group. Titers based on avg values of individual sera.

Virus	No. of sera	Avg titer at		
		37°C	22°C	4°C
Normal mouse brain	2 N*	2	2	24
	2 I †	4	6	48
Encephalomyocarditis	3 N	1	2	18
	3 I	2	10	138
M.M.	2 N	1	1	9
	2 I	5	17	80
Mengo encephalomyelitis	3 N	1	6	37
	3 I	2	20	64
Columbia-SK	3 N	3	8	17
	3 I	3	12	69

* Normal. † Immune.

TABLE II. Cold Hemagglutinins in Rabbit Sera for Human O Erythrocytes both Before and After Immunization with Viruses of the Encephalomyocarditis Group. Titers based on pooled serum specimens.

Virus	No. of sera pooled	Pooled titer at		
		37°C	22°C	4°C
Normal mouse brain	2 N*	Undil.	4	32
	2 I†	2	4	32
Encephalomyocarditis	3 N	Undil.	Undil.	8
	3 I	8	8	128
M.M.	2 N	Undil.	2	2
	2 I	16	32	128
Mengo encephalomyelitis	3 N	Undil.	Undil.	8
	3 I	2	8	8
Columbia-SK	3 N	Undil.	2	8
	3 I	2	8	64

* Normal. † Immune.

Since there was the possibility that normal mouse brain tissue itself might be the inciter of increased cold hemagglutinin production, a control was run with two rabbits which, following the regular cardiac bleeding, were subjected to inoculation with a 10% mouse brain suspension in isotonic saline. In this connection it might be mentioned that Nakamura(6) had found that lipids in raw milk, when injected into normal rabbits, caused increases in the titer of cold hemagglutinins. From the tables it appears that the mouse brain tissue actuated an average 2-fold increase in the titer and some alteration in the thermal amplitude; while the results obtaining from titrations with the same sera pooled manifested no change in titer. For practical purposes then it seems reasonable to assume that any marked changes evinced in the virus-immunized rabbits could be ascribed to the activity of the

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6. Nakamura, I., *Keijo. J. Med.*, 1931, v2, 425.

virus itself.

Further perusal of Table I reveals that EMC virus occasioned approximately an 8-fold increase in titer, M.M. virus a 9-fold increase, and Columbia-SK a 4-fold increase; these changes were accompanied always by augmented thermal amplitudes. In the case of Mengo virus, the increase in titer was of a similar order of magnitude as that following vaccination with the normal mouse brain suspension. In the experiments with pooled sera (Table II) the results were much more striking. A study of the table indicates that EMC virus instigated a marked production of cold hemagglutinins, the immune titer being 16-fold that of the normal, while M.M. virus initiated a 64-fold increase in titer and Columbia-SK an 8-fold increase. Again Mengo virus acted like normal mouse brain, in this case evoking no change.

Discussion. It appears on the basis of these preliminary findings that of these 4 related viruses, M.M., EMC, Columbia-SK, and Mengo encephalomyelitis, the last alone manifests no propensity for provoking the production of cold hemagglutinin.

It appears also from these results that there is a direct relationship between titer and thermal amplitude, a finding in line with those made earlier by Bialosuknia and Hirszfeld(7), and Kettel(8). Hirszfeld(9) was of the opinion, too, that the thermal amplitude reflects also the combining affinity of the antibodies, the agglutinins with greater affinity being less dependent upon conditions of temperature.

The observations of Warren, Smadel, and Russ(4), Dick(10), Olitsky and Yager(11), and Schultz(12) have all pointed to the existence of a generic relationship and common antigenicity among the viruses of the encephalomyocarditis group. While no evidence is presented to the contrary, the findings here might suggest that these viruses may differ in their antigenic make-up insofar as they relate to the production of antibodies against human red blood cells.

The number of variables entering into experiments of this kind suggest, in addition to the use of a larger number of rabbits, employment of inbred animals of the same families and of approximately the same ages. As a further measure to insure against adventitious results, the use of rabbits with the same normal cold hemagglutinin titers would be desirable.

Summary. Cold hemagglutination studies were undertaken to explore the possibilities of this method for neurotropic viral analysis. Preliminary experiments had indicated that such an approach might provide additional differential criteria for the viruses of the encephalomyocarditis family. The observations reported here suggest that while M.M., EMC, and Columbia-SK viruses instigate increased cold hemagglutinin production in rabbits, Mengo encephalomyelitis virus is without such effect. Further work is indicated using larger numbers of animals of more uniform age and genetic constitution.

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8. Kettel, K., *Compt. Rend. Soc. Biol.,* 1929, v100, 371.

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10. Dick, G. W. A., *J. Immunol.,* 1949, v52, 375.

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Received June 22, 1951. P.S.E.B.M., 1951, v77.

Primary Isolation of *Donovania granulomatis* by Chick Brain Inoculation, and Induction of Cerebral Miliary Granulomata. (18848)

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D. granulomatis was cultivated by Anderson(1) in the yolk sac of developing chick embryos. The characteristic lesion of granuloma inguinale has not been reproduced by this organism, although Greenblatt(2), by inoculation of a human volunteer with a yolk sac culture, produced a nodule which after 60 days showed typical Donovan bodies. For yolk sac inoculation, an inoculum free of contaminants must be obtained. This may be accomplished by fragmenting the tissue and incubating it on solid media(1), using for inoculation the fragments which are found to be uncontaminated, or, if possible, by rendering contaminated material sterile by the use of antibiotics to which *D. granulomatis* is resistant.

On the presumption that following direct inoculation of contaminated material containing Donovan bodies into the brains of newly hatched chicks contaminants would not survive, but that *D. granulomatis* might persist, the following experiments were undertaken.

From each of 2 human cases of granuloma inguinale, a 0.5 g piece of granulation tissue, rich in Donovan bodies, was ground and suspended in 5 cc of sterile saline. Routine cultures, consisting of blood agar plates and thioglycolate broth, showed the suspension to be grossly contaminated with Gram positive cocci, Gram negative rods, and diphtheroids. For each case a series of 6 one-day-old chicks were inoculated intracerebrally with .05 cc of the contaminated suspension. After 2 weeks, routine cultures of a brain from each series were sterile, although Donovan bodies persisted on smear. A .5 g piece of brain from a chick of each series was ground and suspended in 10 cc of sterile saline, and .5 cc quantities of this suspension were inoculated into the yolk of a series of 12 five-day-old developing chick embryos. From each strain, growth of a small Gram-negative rod occurred which exhibited bipolar staining and morphological resemblance to *D. granulomatis*. The yolk sac cultures were passed successfully for 3 and 8 generations respectively, and were discontinued; no growth ever occurred on ordinary media.

The remaining 9 chicks in these series were sacrificed at from 14 to 24 weeks, and their brains were examined by smears and by histologic sections. In all of these, smears showed small numbers of organisms morphologically resembling *D. granulomatis*. In 2 examined at 14 weeks, and in one examined at 17 weeks, miliary granulomata occurred in the cerebral hemispheres near the site of inoculation.

It was of interest to reproduce, if possible, these lesions with yolk sac cultures of *D. granulomatis*. Accordingly, a series of chicks were inoculated intracerebrally, using .05 cc quantities of yolk sac cultures from each of the 2 strains above, and also of a culture of a strain previously isolated by the method described by Anderson(1) from a third human case of granuloma inguinale. The brains of 29 chicks were studied by smears and by histologic sections of formalin-fixed material at from 8 to 36 weeks after inoculation. In 6 of these, at from 10 to 27 weeks, miliary granulomata occurred which were similar in all respects to those obtained after inoculation with fresh human material. Organisms morphologically resembling *D. granulomatis* persisted in smears for the full 36 weeks. From three brains so inoculated, at one, 5, and 10 weeks after inoculation, yolk sac cultures were made, yielding organisms morphologically resembling *D. granulomatis*. These were successfully passed for one, 3, and 6 generations, respectively, before being discontinued. Routine cultures were always negative.

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2. Greenblatt, R. B., Dienst, R. B., Kupperman, H. S., and Reinstein, B. S., *J. Ven. Dis. Information*, USPH Service, 1947, v28, 183.

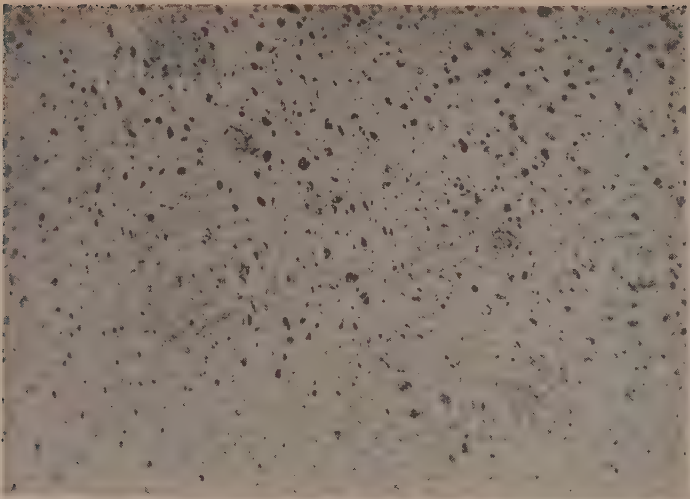

FIG. 1. Section through cerebral hemisphere of chick showing miliary granulomata 17 weeks after inoculation with yolk culture of *D. granulomatis*. H & E stain. × 90.

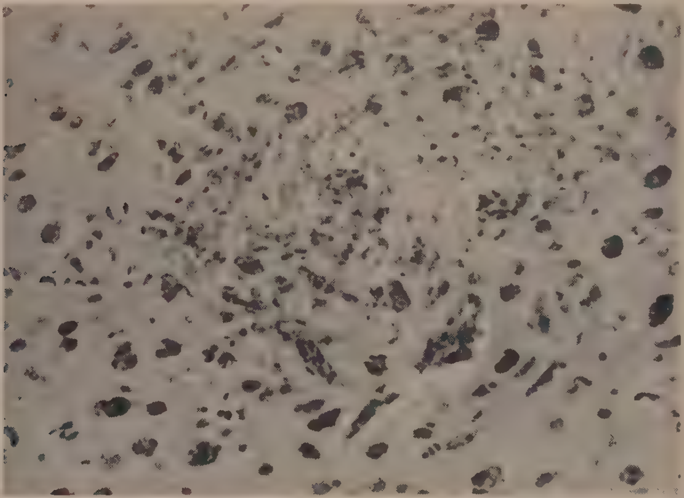

FIG. 2. Section through cerebral hemisphere of chick showing miliary granulomata 17 weeks after inoculation with yolk culture of *D. granulomatis*. H & E stain. × 320.

Gross lesions were never seen in any of the brains, and signs of central nervous system involvement were never observed. Organisms could not be demonstrated histologically in any of the granulomata. A control series of 6 chicks were inoculated with sterile yolk, and no lesions were induced, nor were they induced in a series of 8 suckling mice inoculated intracerebrally with a yolk sac culture of *D. granulomatis*.

Summary and conclusions. 1. *D. granulomatis* was shown to persist for at least 9 months in the chick brain, while contaminating organisms rapidly died out. This finding was utilized in the primary isolation of the organism from contaminated material. 2. Miliary granulomata were induced in the brains of chicks by intracerebral inoculation of material containing *D. granulomatis*.

Received June 25, 1951. P.S.E.B.M., 1951, v77.

Induction of Cataracts in Mice by Slow Neutrons and X-rays.* (18849)

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The causation of cataracts by large doses of X-rays(1,2), fast neutrons(2-5), and by radiation from nuclear explosion ("atomic bomb") (6) is known. It has been established that fast neutrons are more effective in inducing cataracts than X-rays(4), but nothing is known of cataract induction by slow neutrons.

In order to find out the relative efficiency of slow neutrons in producing cataracts, groups of mice of both sexes were placed in the thermal column of the Oak Ridge reactor for 80 minutes (which is about the LD-50 dose), 20, 5, and 2.5 minutes respectively. The slow neutron facilities at the Oak Ridge pile have been described and illustrated by Conger and Giles(7). Mice of the Rf strain were 8 to 10 weeks of age at the time of exposure. Siblings received equivalent doses (on basis of LD-50) of 250 Kv of X-rays, or were untreated. The onset and progression of lens opacities were studied by the slit lamp microscope. The degree of lens opacities was designated + to ++++ as illustrated in Fig. 1. In order to exclude subjective factors, all examinations here reported were made as unknowns. Table I shows the number of mice examined and the degree of opacities observed in relation to time, dose, and kind of irradiation. Examinations on a much large number of animals, not done as unknowns, were in essential agreement with the results shown in Table I.

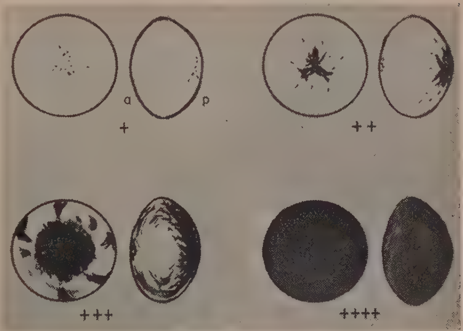

FIG. 1. Degree of opacities of the lens (used for tabulation in Table I).

The earliest change noted consists of small dot-like opacities in the posterior polar region and in the subcapsular layer. They are clustered about the posterior lens suture and appear as an inverted Y-shaped lesion. Further advance of the lesion consists of increased density of the initial opacities and formation of linear opacities radiating into the posterior cortex of lens. In animals exposed to large doses these radiating opacities thicken to form a sunburst pattern extending into the equator of lens and thence around in to the anterior cortical region. Concomitantly, there is the development of anterior polar opacity and anterior subcapsular radiations of a lesser extent than the posterior lesions. The nucleus is spared until the cortical changes are far advanced. Advanced lesions with near total opacity of the lens are noted only in mice of the 80-minute exposure group and only one year after exposure.

In old normal mice cataracts of the type seen in irradiated mice were not observed. There were, however, senile changes consisting of condensation of the lens nucleus, consolidation of the lens capsule, and accentuation of the lens suture. These are not noted in mice examined 1 to 6 months after irradiation, are slight in the 7 to 10 months group, but are

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* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission.

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TABLE I. Incidence of Opacities of Lens of Mice Exposed to Slow Neutrons and X Rays.

Months after exposure	Degree of cataract	Duration of exposure (min) 0	N* 2.5	X† 2.5 (16r)	N 5	X 5 (32r)	N 10	N 20	X 20 (128r)	N 80	X 80 (512r)
6–10	0	37	2	11		4			1		
	±	9	9	5	1	6			3		
	1+	2	5	1	14	6		21	16	2	4
	2+				1			6		20	12
	3+									2	1
14–15	0	11			1	3					
	±	5			9	1	2			1	
	1+	7			8	7	2	1	2	1	
	2+				4		6	6	3		3
	3+									3	4
16–19	0	5	3								
	±	7	2								
	1+	1	4		3	2					
	2+				3	2		3	3		
	3+							2	1	5	2

* N = exposure at a slow neutron flux of .9 × 10^9 per cm^2 per sec with an estimated 4 to 8 r of high energy gamma per min [Conger and Giles(7), Stapleton(8), and Darden and Sheppard(9)].

† X = exposure to X rays at a rate of 6.4 r per min. The factors of irradiation were 250 kvp; 12.5 ma; 3.5 mm Cu; TSD 93.7 cm; rate 6.4 r/min.

consistently present in mice of the 11 to 14 months group. Their presence obscures the opacities induced by slight radiation in mice exposed for 2.5 and 5 minutes. When mice of the 2.5 and 5 minute groups reach about 14 months of age the lesions in their lenses cannot be distinguished from those of normal mice of comparable ages because of the marked senile changes occurring concurrently.

The data here presented indicate that slow neutrons induce lens opacities sooner and in a higher per cent of mice and in smaller doses (on basis of LD-50) than equivalent quantities of X-rays. This difference between the effects of neutrons and X-rays was not evident in: (a) area of lens affected, (b) acute depression of hemopoietic organs, (c) recovery period of erythrogenesis as indicated by reticulocyte counts, (d) weight change after irradiation. In considering the slow neutron/X-ray efficiency ratio the very heavy gamma ray contamination of our slow neutron facility has to be taken into consideration.

It is now generally believed that the dose of X-rays necessary to induce cataracts is large and that men exposed to even as high a dose as LD-50 may remain free from cataracts. The studies of Evans(4) have already suggested that mice are much more susceptible to cataracts than man and the present study indicates that the threshold dose inducing lens opacities in mice is very small, below that required to induce other chronic changes such as leukemia.

Summary and conclusions. The lens of the mouse is extremely sensitive to the induction of opacities by both X-rays and slow neutrons. As small a dose as 32 r of total body X-rays causes a slight increase in the per cent of mice developing opacities of the lens. With larger doses the cataract induction rate approaches 100%, the lesions are more severe, and the latency period is shorter. The morphological features of the radiation and senile cataracts of mice are described. The cataracts develop in the immature part of the lens (cortex), while the more mature part of it (nucleus) is spared. Slow neutrons are, on basis of LD-50, at least twice as effective in inducing cataracts as X-rays.

We acknowledge with thanks the constructive guidance of Dr. J. J. Friedenwald.

Received June 27, 1951. P.S.E.B.M., 1951, v77.

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Serum Sodium Values in Essential Hypertension.* (18850)

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(Introduced by Emmett B. Carmichael.)

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Numerous investigations in recent years have indicated that a relationship exists between the sodium ion and certain hypertensive states(1-4). Beneficial effects of a rice diet in hypertension are thought to be due, at least in part, to a decreased sodium intake(5-7). The effectiveness of sodium restriction in some types of hypertensive cardiovascular disease, while without effect in others, points to a relationship between this cation and the hypertensive syndrome(2,8,9). Selye(2) has shown conclusively that the sodium ion is necessary for the production of experimental hypertension with desoxycorticosterone administration. Likewise, Tobian(4) has shown that this electrolyte is essential to the production of hypertension in certain experimental conditions. Grollman(10) has found an elevated sodium content in various viscera of animals made hypertensive for experimental purposes. Still later, Tobian(11) showed that the sodium content was elevated in the renal artery of hypertensive patients. There have been relatively few reports on the concentration of sodium ion in the serum of hypertensive subjects. Kylin and Elmquist(12) have reported a small series of serum sodium values obtained in hypertensive patients, all of which showed a small but definite elevation from the accepted normal values.

Recently the development of the flame photometer has brought about a simpler procedure for the determination of the sodium concentration in the serum. This method was used to determine serum sodium values in 75 of our hypertensive patients. The purpose of this paper is to present these findings, together with a correlation with those of a normotensive series.

Subjects studied. The outpatient department of the Jefferson-Hillman Hospital offered the source of the patients studied. In this clinic the admissions are made up of Negroes and whites in a ratio of 3 to 1. Sixty-four Negro (⅔ were female) and 11 white patients from the Hypertensive Clinic, who had a diastolic blood pressure reading of 100 mm of mercury or above, were examined. None of these patients presented any symptoms or findings of congestive failure. The ages ranged from 35 years to over 70 years of age, but the majority were between 40 and 55 years. In these patients the diagnosis of essential hypertension was arrived at merely on the basis of exclusion of known causes of hypertension, such as pyelonephritis or other primary renal diseases. These patients had been observed over a period of several years before the diagnosis of essential hypertension was substantiated. No cases of so-called hormonal hypertension as described by Schroeder were examined. Therapy had been discontinued at least 30 days before tests were made. For comparison we used a normotensive series of patients that besides being composed of apparently healthy individuals, were known to have normal blood pressures, and were

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* This work was made possible by a grant from Irwin, Neisler, Decatur, Ill.

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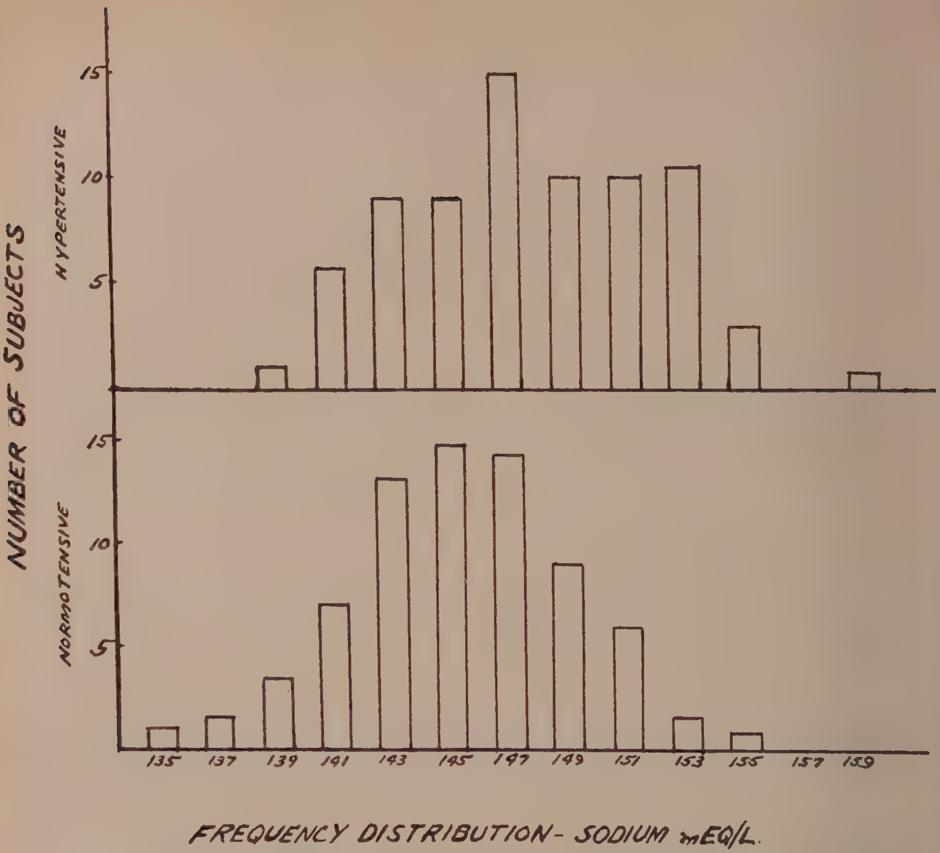

FIG. 1. Frequency distribution curve of serum sodium values in hypertensives as compared to those of normotensive individuals.

otherwise comparable in both sex, race, and age. This normal study was carried out concurrently with the studies on hypertensive patients.

Technic. Blood samples were obtained from nonfasting patients routinely on admission to the clinic and the serum immediately separated. The flame photometric technic used throughout the study was essentially that described by Mosher *et al.*(13). Samples were analyzed in duplicate and the average value of these readings was taken as the correct value. In a series of studies of experimental error it was found that serum sodium can be determined consistently with less than 1% error. The normotensive frequency distribution curve(14), illustrated in Fig. 1, is based on 400 determinations of serum sodium values in 400 different, apparently normal individuals. The technic described above was used. In order to have a basis for comparison with the 75 hypertensive subjects, the number of subjects in each distribution group of the normotensives was multiplied by 75/400 giving the values plotted in Fig. 1.

Results. The mean value for "normal" serum sodium, as determined by this work, was 144.8 mEq/1. The mean value found in

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75 hypertensive patients was 147.3 mEq/1, an increase of 2.5 mEq. A statistical analysis based on 400 normotensive determinations and 75 hypertensive determinations using Fisher's "t" test(15) showed the difference between the means to be significant. The value of "t" was 6.16 and that of "P" was less than .01. The standard deviation in the 400 normal determinations was 3.81 mEq/1, and the standard error 0.19 mEq/1. In the 75 hypertensive patients the standard deviation was 4.07 mEq/1, and the standard error was 0.45 mEq/1. In the frequency distribution curves shown in Fig. 1 it is obvious that there is a tendency toward an increase in the sodium values present in the serum of hypertensive individuals. Forty-three per cent of "normal" values fall to the right of the normal mean on the curve, while 66% of the hypertensive values fall in these same limits.

Twenty-four of the patients were recalled for further examination two months after the initial values were obtained. This was thought necessary in order to determine the constancy of the values previously found. Ninety-two per cent of the values checked were within plus or minus 0.5%. We believe that these deviations were within the range of technical error.

Discussion. The results obtained would seem to indicate that there is a tendency toward an elevation in the serum sodium concentration of hypertensive patients. Surprisingly enough, statistically this elevation was found to be very significant. These data indicate a wide overlap between the serum sodium values found in normotensive and hypertensive patients, with a definite trend for a large group of the hypertensives to have a value in the upper normal range. One might be tempted to conclude that this finding supports the concept of adrenal participation in the production of the hypertensive syndrome, and that this might offer a basis for sodium restriction therapy in this disease. However, until the significance of these findings is more clearly understood, it may perhaps be better to avoid any conclusion concerning these findings as to their therapeutic or etiologic concepts.

Summary and conclusions. A series of serum sodium determinations made on uncomplicated hypertensive patients has been presented. The range and average serum sodium values in these patients were found to be slightly above those found in a concomitant series of 400 normotensive subjects. We have intentionally avoided any conclusions at this time as to the significance of these findings.

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Received March 30, 1951. P.S.E.B.M., 1951, v77.

Apoerythein Studies. IV. Nonidentity of Salivary, Gastric Juice, and Gastric Mucosal Apoerythein Activities. (18851)

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Previous papers in this series have reported the normal variations which occur in salivary apoerythein (intrinsic factor) levels(1) and demonstrated the unique manner in which heating affects salivary apoerythein activity (2,3). The heating of saliva at 70°C for 15 seconds results in a complete loss of apoerythein activity, but heating below 65°C *or at 75°C-100°C* results in no diminution of activ-

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1. Beerstecher, E., Jr., and Altgelt, S., *J. Biol. Chem.*, 1951, v189, 31.

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TABLE I. Comparison of Thermal Stabilities of Apoerythein Activity of Saliva, Gastric Juice, and Gastric Mucosal Extract.

Sample	Temp., °C	% bacterial growth* Milliunits apoerythein/5 ml medium			
		0.2	0.4	0.6	0.8
Human saliva	Untreated	100	25	20	0
	70°	100	110	120	140
	90°	100	75	40	0
,, gastric juice	Untreated	100	35	25	0
	70°	100	160	180	220
	90°	100	160	210	225
Dog ,, ,,	Untreated	100	90	55	0
	70°	100	105	120	125
	90°	100	100	125	140
Dog ,, mucosa	Untreated	100	15	0	0
	70°	100	60	15	0
	90°	100	90	30	0
Hog ,, ,,	Untreated	100	85	35	0
	70°	100	85	45	0
	90°	100	85	65	0

* 0 = no growth; 100 = growth of bacteria in absence of apoerythein.

ity. Samples inactivated at 70°C cannot be reactivated by subsequent heating at higher temperatures, but samples heated above 75°C become immune to destruction at 70°C. Thermal studies with apoerythein from gastric juice and gastric mucosa now show that the activity differs from that in saliva.

Experimental. The apoerythein assay method was that described by Ternberg and Eakin(4) in which activity is indicated by the inhibition of the growth of *Escherichia coli.* Samples of human saliva and gastric juice, dog gastric juice and gastric mucosal extract, and hog gastric mucosal extract were diluted to contain approximately one milliunit of apoerythein per ml and immersed in a water bath until the desired temperature was reached. They were held at this temperature for 5 minutes, then cooled. Different aliquots of each sample were then assayed, and the results reported in terms of the percentage of bacterial growth obtained in the absence of apoerythein. Results are reported in Table I. A greater variety of temperatures was studied than is shown, but the effects are adequately represented by the data given for room temperature materials and those heated at 70°C and 90°C.

The data indicate clearly that the apoerythein activity in each of the 3 sources studied has a different thermal behavior. Gastric juice activity disappears when the sample is heated to 70°C or higher while mucosal extract activity is only slightly decreased at 70°C or higher. Mucosal activity thus resembles that of saliva which has been heated to 90°C in its ability to withstand heating at 70°C.

Discussion. Three possibilities present themselves in explanation of the data presented in this and earlier publications(2,3). The apoerythein molecule may undergo true variations in its configuration and activity upon exposure to various higher temperatures. It is also possible that at 70°C a substance is formed from salivary apoerythein which is capable of neutralizing its normal inhibitory effect in the assay. Also, some entirely foreign factor in the saliva may be activated sharply at 70°C to inactivate apoerythein, and this substance may be inactivated at higher temperatures. Attempts to exclude some of these possibilities have to date been unsuccessful in that highly suggestive evidence has been found to support each possibility.

Summary. Salivary apoerythein activity is destroyed at 70°C, *but not* at higher temperatures up to 100°C; gastric juice apoerythein activity is destroyed at 70°C *and* at higher temperatures up to 100°C; but gastric mu-

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cosal activity *is not* destroyed appreciably at any temperature up to 100°C.

The authors are grateful to the Lilly Research Laboratories for a generous supply of ground desiccated hog gastric mucosa; and to Mrs. Suzanne Beerstecher for supplying numerous samples of human gastric juice from both healthy and pathological individuals.

Received April 30, 1951. P.S.E.B.M., 1951, v77.

Action of Amino Acids on Bacterial Dehydrogenases and Glycolysis. (18852)

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In a previous communication(1) it was shown that in a system containing washed resting *Micrococcus pyogenes* var. *aureus* and glucose the reduction of triphenyltetrazolium was greatly accelerated by nitrogenous substances. Effects of amino acids on biological systems have been variously reported. Amino acids were found to increase markedly the motility, fertilizing power and agglutination ability of starfish sperm(2), greatly to extend the functional life span of sea urchin spermatozoa(3) and under anaerobic conditions to induce motility in these spermatozoa which are otherwise quickly immobilized by lack of oxygen(4). In these studies no amino acid utilization was reported to occur.

The effect of amino acids observed under our conditions was subjected to quantitative analysis to characterize the underlying mechanism.

Materials and methods. *Micrococcus pyogenes* var. *aureus* strain 1A was carried on extract agar slants kept at 4°C. The cells washed from a slant which had been incubated at 37°C for 20 hours were used as source of inoculum. A casein hydrolyzate medium containing added glucose (0.5%), tryptophan (20 μg/ml), thiamin (1 μg/ml), and nicotinamide (1 μg/ml) was inoculated with 250,000 cells/ml, and when growth had reached the middle of the log phase, the cells were collected and washed twice with 0.066 M phosphate buffer (pH 7.0). The amount of cells used in the reaction systems was determined by comparing the turbidity of the systems with a previously calibrated curve relating turbidimetric readings (filter 56) and the dry weight of cells. The glucose content of the samples removed from the reaction systems at the start and at the end of the experiment was determined by the method of Folin and Wu(5). Under the conditions of the experiment triphenyltetrazolium, formazan and amino acids did not interfere with these determinations. Lactate was determined by the method of Barker and Summerson(6). The copper-lime treatment removed glucose, tetrazolium, histidine and alanine so that these substances did not interfere with the determinations. The red formazan formed by the reduction of triphenyltetrazolium chloride was measured by the use of the method described by Kun and Abood(7). Acetone (7 ml) was added to a 4 ml sample, the mixture shaken, centrifuged, and the supernate read on the Klett-Summerson colorimeter (filter 42). The quantity of formazan was determined by reference to a standard curve relating known concentrations of tetrazolium which had been reduced with a few crystals of sodium hydro-

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* Abbott Research Fellow.

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TABLE I. Effect of Various Compounds on the Reduction of Tetrazolium by *Micrococcus Pyogenes* var. *Aureus.*

Systems (1A)*	Colorimetric reading†	Systems (1B)*	Colorimetric reading†
No addition	11	Glycine	165
Glycine	90	Pyruvate	21
Ethylamine	65	α-oxo-glutarate	20
Sarcosine	50	Acetate	22
Betaine	53	Benzoic acid	18
Choline	24	Salicylate	24
PABA	33		
Ammonium chloride	35	Cells + glycine + TTZ	0

* (1A and 1B). Each system consisted of 6 ml volume. Each ml contained 30 μg cells, 500 μg glucose, 100 μg tetrazolium dissolved in 0.067 M phosphate buffer (pH 7.05). Concentration of substances added in Systems (1A) was 0.0025 M; in (1B) 0.005 M.

† Colorimetric readings (filter 54) indicate increment in red color due to formazan formation after incubation of 120 min at 37°C. Readings corrected for zero hr readings.

TABLE II. Effect of Amino Acids and Tetrazolium on Glycolysis by *Micrococcus Pyogenes* var. *Aureus.*

Systems*	μMol of glucose utilized	μMol of lactate formed	Molar ratio glucose/lactate	μMol of formazan formed
1. Cells + glucose	.55	.17	3.2	—
2. ” ” ” + histidine†	.55	.27	2	—
3. ” ” ” + TTZ	.13	.11	1.2	.005
4. ” ” ” + histidine + TTZ	.33	.16	2	.11
5. ” ” ”	.90	1.13	.78	—
6. ” ” ” + histidine	.90	1.35	.66	—
7. ” ” ” + TTZ	.90	1.07	.84	.18
8. ” ” ” + histidine + TTZ	.90	1.13	.78	.18
9. ” ” histidine + TTZ	—	—	—	0
10. Heat killed cells + glucose + histidine + TTZ	0	0	—	0

* Each ml of the 30 ml system contained 40 μg (Systems 1-4) and 80 μg (Systems 5-10) cells, 2.77 μMols glucose, 0.32 μMols histidine, and 0.29 μMols of TTZ, as indicated, which were dissolved in 30 ml of 0.066 M phosphate buffer (pH 7.05). At start of experiment 15 ml were removed and inactivated by heating in boiling water, the remaining 15 ml were incubated for 3 hr at 37°C in 50 ml centrifuge tubes, then similarly inactivated. The pH measured in Beckman pH meter remained constant. Formazan was determined by the method of Kun and Abood(7).

† Methionine and alanine gave similar results. Chemical analysis for histidine showed that, if any, <1 μg/ml was utilized in the 3 hr experiment. Microbiological determination for methionine showed that, if any, <1 μg/ml methionine was metabolized.

sulfite and the colorimetric readings of their acetone supernate. A more sensitive method used by us consisted in reading the red color due to formazan formation directly in the reaction system. This method permitted us to follow on a comparative basis the effect of various factors on the progress of the reduction of tetrazolium in a constant volume. It could be used when the amount of cells (10 to 40 μg/ml) used in the experiment was small and remained in a homogeneous suspension throughout the experiment. Histidine was determined by the modification of the method of MacPherson(8) as described by Straughn and Sevag(9). This method permitted the determination of 1 μg of histidine, but did not allow differentiation between histidine, histamine and the keto derivative of histidine. Methionine was determined microbiologically in usual salt-glucose medium by means of an *E. coli* mutant kindly furnished by Dr. J. S. Gots of this department. This method permitted the detection of 1 μg of the amino acid. Anaerobic experiments were performed in Thunberg tubes which fitted into the Klett-Summerson colorimeter. The tubes attached to a manifold were evacuated to a residual

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8. MacPherson, H. T., *Biochem. J.*, 1942, v36, 59.

9. Straughn, W. R. and Sevag, M. G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 360.

pressure of 10-15 mm Hg by means of a high vacuum pump, equilibrated in the water bath at 37°C for 5 minutes, after which the content of the side arm was mixed and colorimetric readings made periodically.

The results of the single experiments presented are representative of a series of experiments the results of which were closely similar. All determinations were carried out in triplicate.

Results. Amino acids while unable to serve as substrates for the reduction of tetrazolium by resting cells of *Micrococcus pyogenes* var. *aureus* are the most effective of the compounds tested (Table I) in accelerating the reduction when added to the reaction system in combination with glucose.

Due to a greater reaction rate, the effect of the amino acid on the amount of lactic acid formed is masked when a larger amount of cells is used[†] (Table II, compare systems 1 and 2 and 5 and 6). This is indicated by the fact that the accelerative effect of amino acids on the reduction of tetrazolium in such systems can only be shown during a very early phase of reaction (compare systems 3 and 4 with 7 and 8, Table II). With smaller concentrations of cells the presence of the amino acid can be shown to cause 50% more lactate formation (in other experiments not reported here as high as 300% more lactate production was observed) and to cause the reduction of 20 times more tetrazolium than in the absence of the amino acid (Table II, systems 1 and 2, 3 and 4).

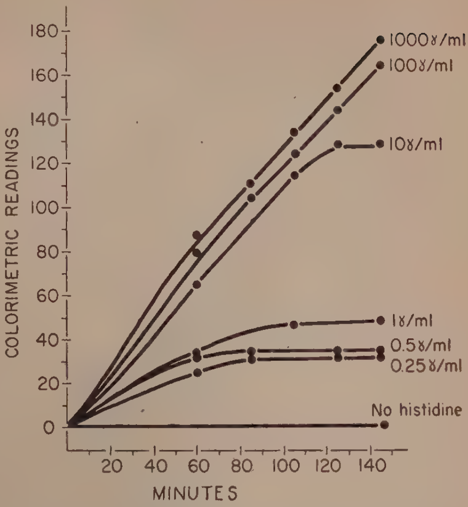

FIG. 1. Reaction systems in Klett tubes consisting of 8 ml volume. Each ml contained 40 μg cells, 500 μg glucose, 100 μg of tetrazolium, histidine.HCl as indicated dissolved in 0.067 M phosphate buffer (pH 7.30) and incubated at 37°. The colorimetric readings (filter 54) represent increment in red formazan formation and are corrected for the small zero hr readings.

The rate of reduction and the amount of formazan formed may appear to be proportional to the concentration of the amino acid added (Fig. 1); the relationship, however, is not a stoichiometrical one. The quantity of amino acid utilized, if any, must be less than 1 μg/ml (0.0064 μm histidine) and since the quantity of tetrazolium reduced is in the order of 40 μg/ml (0.11 μm) (Table II, systems 3 and 4) the action of the amino acid might be catalytic in nature. No disappearance of histidine could be demonstrated. The method was shown to permit the detection of the disappearance of 1 μg of histidine from a system of 6 ml containing 10 μg of histidine. Also in microbiological assay using a methionineless *E. coli* mutant, we failed to demonstrate any disappearance of methionine from the medium during the reaction. Control experiments showed that this method would detect the removal of as little as 1 μg of methionine per ml. The fact that oxidation products of the amino acids, the keto acids, and the ammonium ion have only a slight effect (Table I) and the variety of nitrogenous substances (*e.g.*, sulfathiazole, ethylamine, etc.) causing some degree of acceleration of the reduction of tetrazolium, tends to eliminate utilization as a mechanism for the observed effects of amino acids.

Tetrazolium chloride acting as an unnatural hydrogen acceptor inhibits glucose metabol-

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[†] Since in Warburg set-up satisfactory measurements of gas exchange require at least from 500 to 1,000 μg cells/ml, its use for the study of the reported effect of amino acids could not be adapted. In contrast, the use of tetrazolium in place of oxygen as hydrogen acceptor permitted the measurement of dehydrogenase activity of as little as 20 to 40 μg cells/ml.

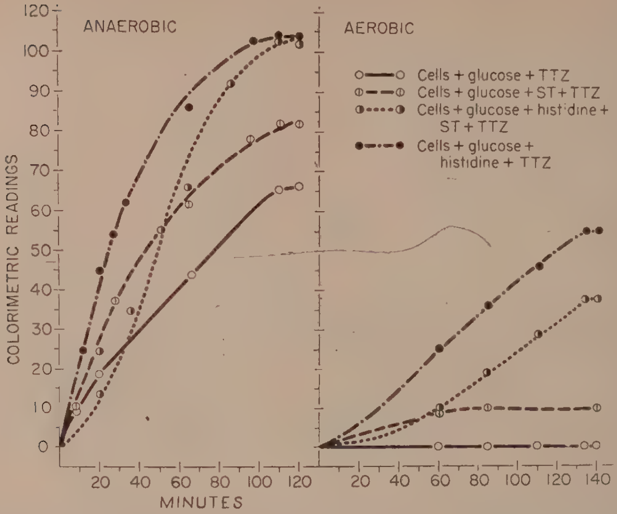

FIG. 2. Anaerobic systems in Thunberg tubes consisted of 6 ml volume. Each ml contained 20 μg of cells, 500 μg sulfathiazole (where added) in the main compartment, and 500 μg glucose, 100 μg tetrazolium, 100 μg histidine.HCl (where added) in the side arm dissolved in 0.067 M phosphate buffer (pH 7.30). The tubes were evacuated to a residual pressure of from 10-15 mm Hg, equilibrated for 5 min at 37°, after which the contents of the side arm were mixed and periodical readings taken in the colorimeter using filter 54. Aerobic systems in 50 ml centrifuge tubes consisted of 6 ml volume. Each ml contained 40 μg of cells, and was otherwise identical with the systems used in the anaerobic experiments. To insure good aerobic conditions the mixtures were periodically pipetted into Klett tubes, at which times the color increment was read on the colorimeter. No formazan was formed aerobically or anaerobically in systems containing only cells, histidine and tetrazolium.

ism (Table II, systems 1 and 3); this inhibition is not apparent when larger concentrations of cells are used. The specific effect of the amino acids is partially to relieve the inhibition of the oxidative enzymes involved in the metabolism of glucose (Table II, systems 3 and 4).

Inhibition of the reduction of tetrazolium. Comparison of parallel aerobic and anaerobic systems show that oxygen is probably competing with tetrazolium as a hydrogen acceptor indicated by the inhibition of the reduction of tetrazolium by resting cells in the presence of glucose (Fig. 2). Sulfathiazole slightly accelerates the reduction of tetrazolium when glucose alone is a substrate. The acceleration by amino acids of the reduction of tetrazolium aerobically or anaerobically is however inhibited by sulfathiazole, showing that the drug interferes with the effect of the amino acids on dehydrogenases.‡

Discussion. Flavoprotein enzymes have been reported to be the site of reduction of tetrazolium(10-12). In view of the striking effect of the amino acid on the reduction of tetrazolium, and lactic acid production from glucose, it would seem reasonable to assume that this action of amino acids is related to their interaction with flavoproteins. This view is corroborated by the partial relief by amino acids of the inhibition of glucose consumption by tetrazolium. Similarly, the gradual decrease in the sulfathiazole inhibition of the amino acid accelerated reduction of tetrazolium can be explained by a gradual displacement by amino acids of the drug from its site of action on flavoprotein. The inhibitory action of sulfonamides on flavoprotein has been abundantly reported(13).

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‡ The inhibitory effect of α-oxo-glutaric acid reported previously(1) was due to inadequate neutralization of compound and consequential lowering of pH of medium.

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The results of the parallel aerobic and anaerobic experiments therefore show that oxygen, tetrazolium and amino acids, and tetrazolium, sulfathiazole and pyruvate compete for the same enzyme site.§

In the absence of evidence showing utilization of the amino acids, the observed effects of these substances appear to be contingent upon the inhibition of reactions competing for the available hydrogen. As to the nature of these competing reactions it is of interest to note that Meyerhof and Schulz(14) found that only upon addition of certain nitrogenous substances (certain amino acids, amines or ammonium sulfate) to fermentation mixtures did yeast completely convert hexoses to CO_2, because under these conditions assimilation of sugar was restricted. In the absence of added nitrogenous substances only 75 to 80% of the theoretical yield of CO_2 was obtained.

Summary. Amino acids, aerobically and anaerobically, cause an acceleration of the reduction of tetrazolium by *Micrococcus pyogenes* var. *aureus* in the presence of glucose, and can be shown to shift glucose metabolism towards increased lactate production. This effect of amino acids appears to be related to the inhibition of normally occurring reactions resulting in an increased availability of hydrogen for the reduction of tetrazolium or pyruvate.

Oxygen inhibits the reduction of tetrazolium in the absence of added amino acids, while sulfathiazole inhibits the amino acid accelerated reduction of tetrazolium. Since flavoprotein is reported to mediate tetrazolium reduction and to be susceptible to inhibition by sulfathiazole, the accelerative effect of amino acids on the reduction of tetrazolium could be related to an interaction of the amino acids with the flavoprotein enzyme. Competition for this enzyme site between tetrazolium, oxygen and pyruvate, modified by the presence of amino acids and sulfathiazole is suggested as an explanation consistent with the observed accelerations and inhibitions of the reduction of tetrazolium.

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13. Sevag, M. G., Gots, J. S., and Steers, E., *The Enzymes,* Vol. I, New York, Academic Press, 1950, pp115-186.

§ The alternative postulate that the amino acid acts by lowering the oxidation-reduction potential of the medium to a level more favorable for tetrazolium reduction does not appear to find support in the fact that all the amino acids and other nitrogenous substances tested are active in low concentrations and under strictly aerobic and anaerobic conditions under which O/R potentials would widely vary.

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Received May 3, 1951. P.S.E.B.M., 1951, v77.

Protein Depletion Enhances Pancreatic Damage Caused by Ethionine. (18853)

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Destruction of pancreatic tissue by the administration of ethionine in the rat has been recently reported by Farber and Popper(1) and by Goldberg, Chaikoff, and Dodge(2). In an attempt to reproduce the results of these investigators pancreatic damage was found to be slight and seen only irregularly in rats on a complete diet. It is the purpose of this communication to describe a procedure by which pancreatic damage can be regularly produced by simply putting the rats on a protein depletion regime prior to the administration of the drug.

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2. Goldberg, R. C., Chaikoff, I. L., and Dodge, A. H., Proc. Soc. Exp. Biol. and Med., 1950, v74, 869.

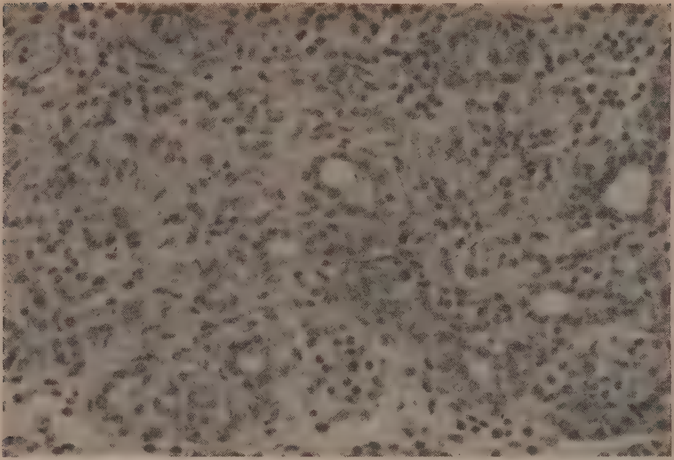

FIG. 1. Photomicrograph of pancreas. Hematoxylin-eosin stain × 250. Rat was kept for 12 days on the protein depletion diet prior to ethionine administration; sacrificed 6 days later. There is extensive replacement by proliferating fibrous tissue present. Only few acini are preserved. Small ducts show dilated lumina.

Experiments and results. Young male albino rats of the Wistar strain were used. Of 11 rats, on a complete Rockland diet, weighing between 120 and 250 g, 8 received 1 mg of ethionine (α-amino-γ-ethylthiolbutyric acid) (Nutritional Biochemical Corporation, Cleveland, Ohio) per g body weight given in 3 divided doses intraperitoneally after a fast of 16 hours as suggested by Farber and Popper(1). In 3 animals this procedure was repeated on 3 consecutive days and the rats were killed 3 to 5 days later. In all instances there was infiltration of the peri-pancreatic fat tissue by mononuclear cells and occasional polymorphonuclear leucocytes. Degenerative changes within the pancreas, however, were either entirely absent, or only of moderate degree often restrictd to focal isolated areas. It was considered possible that thorough protein depletion prior to the administration of the drug might lead to more consistent results. Rats were therefore put on a protein depletion diet consisting of 77.6 parts corn starch, 15 parts hydrogenated cotton seed oil (Crisco), 4 parts salt mixture No. 2 U.S.P. XII, 4 parts Ruffex, and 0.4 parts Brewers yeast powder. In addition the animals received twice weekly one drop of cod liver oil. Within 10 to 14 days these rats lost an average of 22% of their initial body weight. In all experiments 1 mg of ethionine per g body weight was given in the same manner as previously mentioned, however, without preceding fast. Altogether 56 rats weighing between 140 and 215 g were used of which 3 died within 72 hours following the administration of ethionine.

It was found in preliminary experiments that the rats had to be kept on the protein deficient diet for a minimum period of 5 days in order to increase the pancreas damaging action of the drug. Thirty-three rats were therefore given the protein depletion diet for a period of 7 to 19 days and killed 1 to 7 days later.

The microscopic appearance of the damaged pancreas was essentially similar to that described by Farber and Popper(1), and Goldberg, Chaikoff, and Dodge(2). After 24 hours there was loss of basophilia and marked swelling of the cytoplasm. In the subsequent days there was prominent vacuolization of the cytoplasm while coagulation necrosis was only occasionally noticed in our material. Nuclei were little changed. Infiltration by polymorphonuclear leucocytes, eosinophiles and lymphocytic elements became pronounced on the third day. There was considerable proliferation of fibroblasts commencing on the third to fourth day. Strands of proliferating fibro-

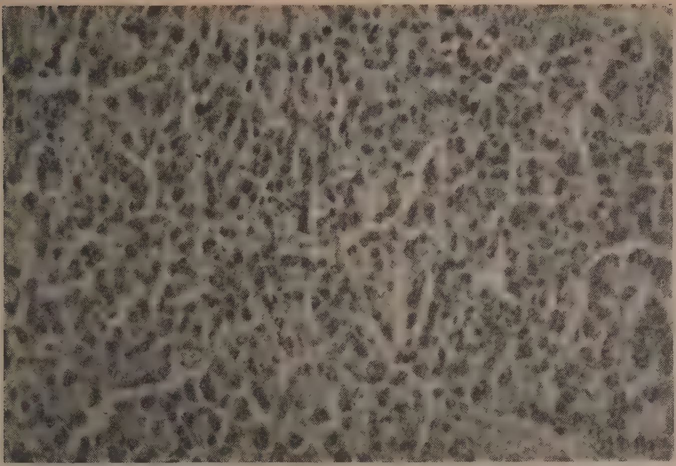

FIG. 2. Photomicrograph of pancreas. Hematoxylin-eosin stain × 250. Rat was sacrificed after 17 days on the protein depletion diet. There is focal depletion of zymogen granules present.

blasts separated remaining pancreatic acini. These acini showed basophilia of the cytoplasm but very few zymogen granules. Langerhans islands remained intact but pancreatic ducts were often dilated. While in some instances the changes were only focal, in most they were diffuse and were estimated to involve up to ¾ of the acinar tissue on representative sections (see Fig. 1). In sections of the pancreas of 6 control rats kept for 11 to 18 days on the protein depletion diet, focal moderate zymogen degranulation was seen(2). There was no loss of basal and perinuclear basophilia. No comparable destructive or inflammatory changes were observed. (See Fig. 2).

The livers of some of the rats which had been given ethionine showed the histological picture of protein depletion as described by Elman, Smith, and Sachar(3). In these instances the liver cells appeared enlarged with prominent cell walls and rarified cytoplasm with no or very little stainable fat. In other livers, however, the cells were small and showed diffuse fatty infiltration(4).

Comment. The decrease in resistance to the pancreas damaging action of ethionine in young male rats after protein depletion is very striking. It is interesting to note that the susceptibility of the pancreatic islands to the toxic action of alloxan is influenced by age and dosage level(5) as well as by the preceding diet. Houssay and Martinez(6) have shown that the toxic and diabetogenic action of alloxan increases in rats fed a low protein diet and even more in the case of a high lard or ox-fat diet. Starvation for a period of 48 to 60 hours made rats likewise considerably more susceptible(7).

High activity of radioactive sulphur found in the pancreas of normal rats after the administration of labelled methionine is thought to represent principally the metabolic activity of the exocrine portion of the pancreas(8). Since ethionine presumably interferes with the utilization of methionine(1) the increased susceptibility of the rat pancreas under the experimental conditions described can be satisfactorily explained by the depletion of tissue

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4. Jaffe, E. R., Humphreys, E. A., Benditt, E. P., and Wissler, R. W., *Arch. Path.*, 1949, v47, 411.

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7. Kass, E. H., and Waisbren, B. A., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 303.

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protein prior to the administration of the drug.

Summary. Ethionine given to young male rats of the Wistar strain on a complete diet caused only inconsistent and moderate pancreatic damage. Rats that had been on a protein depletion diet for a period of at least 6 days showed extensive destruction of the pancreas following the administration of equal amounts of the drug.

Received May 11, 1951. P.S.E.B.M., 1951, v77.

Successful Subcutaneous Growth and Transplantation of Human Tumors in X-Irradiated Laboratory Animals.* (18854)

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Neoplasms fail to grow in heterologous hosts unless one or both of two conditions are met. Either the tumor is one which has been transplanted for many generations or the implantation is made in sites at which the host offers minimal resistance to foreign tissue. Examples of these sites are the anterior chamber of the eye, brain, testes and immature tissues such as avian embryos and newborn animals. Spontaneous neoplasms, such as those of man, have been propagated heterologously only when implanted in the anterior chamber of the eye(1). The percentage of failures has been high, even under these circumstances, the rate of growth of the tumors slow, and the transplantability poor(2). To achieve an improved method for growing human neoplasms in animals, the work here reported was undertaken

Murphy showed(3) that rats which had been X-irradiated would support the growth of subcutaneously implanted mouse tumors for the duration of the lymphopenia produced by the radiation. A modification of this procedure has been employed in our experiments.

Methods and materials. Young adult female albino rats (Carworth Farms) and mice (Banks strain of Swiss) were employed. The rats received 3 doses each of 200 r, total body X-irradiation, on alternate days and the mice 4 doses of 150 r on successive days. Two 180 kv. tubes were employed (one above, one below the animal).

Previously, it had been established that this procedure would reduce the total white count in the particular strains of animals used, to approximately 400-800 mm^3. Several extra animals were included in each group irradiated to check the degree of lymphopenia produced. Counts were *not* made on the animals to receive implants since too great a variable is introduced by the cardiac puncture required to obtain a true picture of the total white count of rodents; tail blood is not usable because of its hemoconcentration.

The divided irradiation doses caused no symptoms of toxicity in the animals whereas a single dose large enough to produce the same reduction of white cells often caused diarrhea and even an occasional death. It must be em-

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* We wish to acknowledge support of this study by funds from the National Cancer Institute, Public Health Service, Institution of Research Grants, American Cancer Society and the Damon Runyon Memorial Fund for Cancer Research.

Our gratitude is expressed to Dr. C. P. Rhoads for his faith and encouragement; to Drs. Stewart, Foote and Sunderland of the Pathology Department, Memorial Hospital, for their cooperation in the procurement of human tumors; to Miss Priscilla Goettler who grew the original tumors and transplants in tissue culture; and to Dr. M. Harland for the considerable histological material prepared.

1. Hegner, C. A., *München Med. Wchnschr.*, 1913, v60, 2722; Greene, H. S. N., *Science*, 1938, v88, 357; *Canc. Res.*, 1942, v2, 649; with Lund, P. K., *Canc. Res.*, 1944, v4, 352; *Yale J. Biol. and Med.*, 1950, v22, 611.

2. Schilling, J. A., Snell, A. C. and Favata, B. V., *Cancer*, 1949, v2, 480. Towbin, A., *Canc. Res.*, 1951, v11, 286 (abst).

3. Murphy, J., *J. A. M. A.*, 1914, v62, 1459.

TABLE I. Results of Subcutaneous Implantation of 100 Human Tumors into X-irradiated Rats and Mice.

No. of tumors	Pathological diagnosis	Mitosis for more than one generation	Mitosis at end of 1st generation (8 days) only	Failed
I – Malignant				
48	Epidermoid Ca.	21	15	12 (6 infected)
10	Ca. breast	1 (poor)	3 (poor)	6 (1 ”)
10	Ca. colon, sigmoid or rectum	1	2 ”	7 (4 ”)
4	Melanoma	1 ”	2 ”	1 (”)
3	Ca. ovary	1 ”	2	—
3	Osteogenic sarcoma	2	—	1
2	Leiomyosarcoma	1	1	—
2	Fibrosarcoma	1	1	—
2	Synovioma	1	1	—
2	Rhabdomyosarcoma	1	—	1
2	Ca. lung	—	1 ”	1
1	Ca. prostate	1	—	—
1	Malignant mixed tumor parotid	1	—	—
1	Fascial sarcoma	—	1 (not transferred)	—
1	Ca. adrenal cortex	—	1 (poor)	—
1	Ca. kidney (“clear cell”)	—	1	—
1	Angiosarcoma	—	—	1
1	Wilms	—	—	1 (”)
1	Ca. uterus	—	—	1
96		33*	31	32
II – Benign				
2	Mixed tumor parotid	—	2	—
1	Hemangioma	—	1	—
1	Leiomyoma	—	—	1
4		0	3	1
Total: 100		33	34	33

* Of 33 tumors in which mitotic cells were present for more than one generation, 18 showed active mitosis at end of the 2nd generation, 9 at end of the third, and 6 had actively growing cells at end of the 4th generation (approximately a month after the original tumors were obtained); one epidermoid carcinoma was transferred successfully for 9 generations.

phasized that different strains of the same species of animal may vary greatly in their susceptibility to radiation. This factor must be determined carefully for each strain used.

Samples of human neoplastic tissue were implanted 1-8 hours after removal in X-irradiated animals which had received their 3rd, and final treatment 1-3 days previously.

Of the 100 tumors implanted, the first 47 were pressed through a 40 mesh monel metal sieve into a sterile Ringer's solution buffered to a pH of 7.35 (3 mg of glucose and 200 units of penicillin added per cc). Sieving was employed to insure suspensions of cells with relatively few clumps for implantation. As a result, any large masses of tumor subsequently found at the implantation sites could be ascribed to multiplication of cells rather than survival of a graft *in toto*. Each of the next 6 tumors was divided into two parts. One was sieved as usual, the other minced fine enough with scalpels so that the suspension would pass through a No. 20 needle. The remaining 47 tumors were minced only. All the tissue transplanted beyond one generation was minced.

With few exceptions each tumor suspension was implanted subcutaneously in both X-irradiated mice and rats, usually in groups of 6 to 10 each. Mice were given 0.5 cc of the concentrated suspension in each flank. The rats were either implanted in similar manner or in 4 sites, both groins and both axillae. On the 8th day after implantation (occasion-

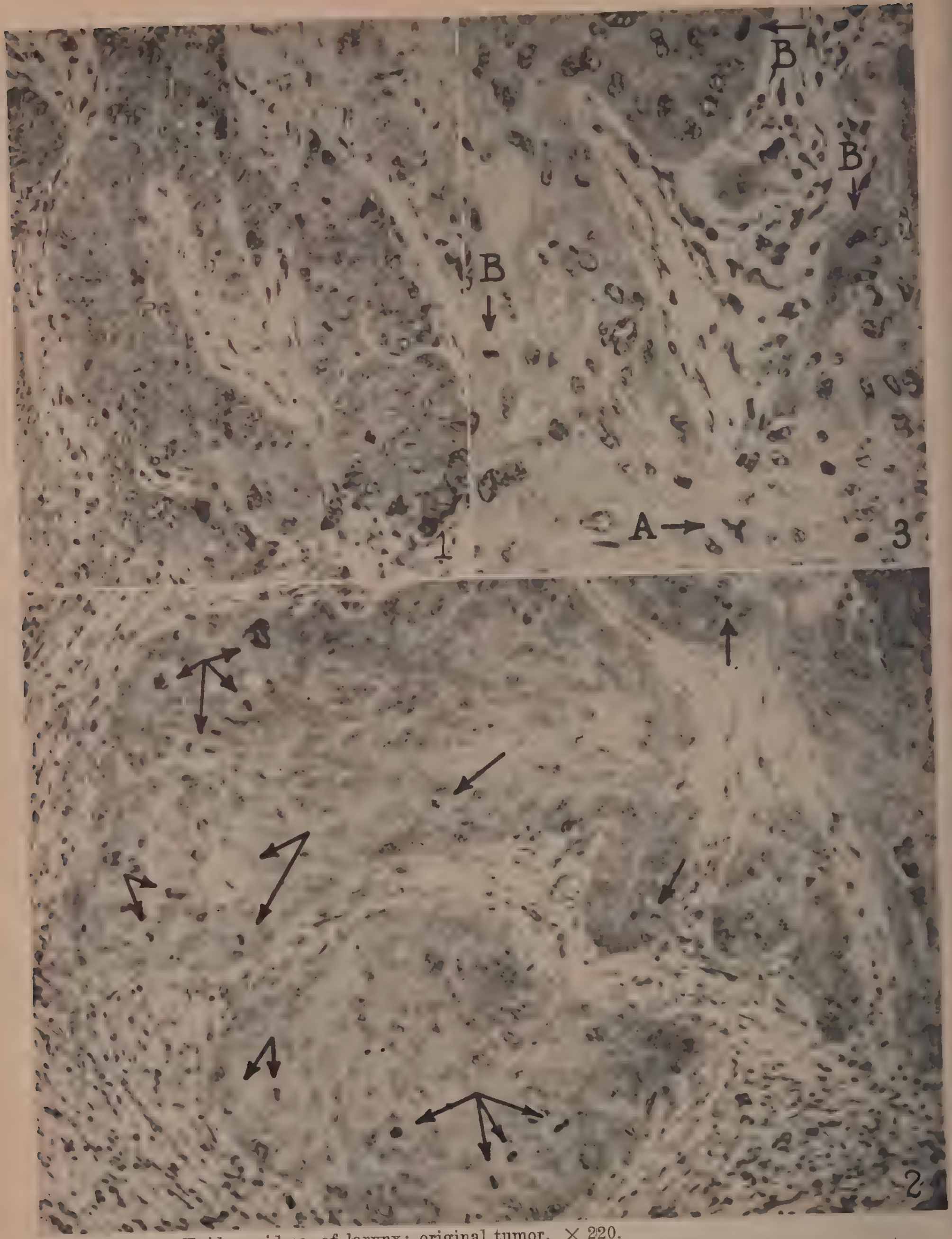


FIG. 1. Epidermoid ca. of larynx; original tumor. × 220.

FIG. 2. Same tumor as in Fig. 1 after 3 generations (25 days) in X-irradiated rats; note the many mitotic figures (arrows). Original implantation a sieved suspension of cells. × 200.

FIG. 3. Same tumor as in Fig. 1 after 3 generations (25 days) in X-irradiated mice; note tripolar mitosis (arrow A) and other mitotic figures (arrows B). Original implantation a sieved suspension of cells. × 350.

ally on the 7th or 9th) the animals were killed and the nodules at the sites of implantation were removed, samples taken for histological examination, and the remainder pooled, minced and reimplanted in a new group of irradiated animals. After 8 days the procedure was repeated—the number of subsequent transfers varying with the tumor.

Choice of the 8th day as an optimum time for transplantation was due to the finding that the ratio of implanted tumor cells in good condition to the amount of host reactive tissue was most favorable to the tumor at this time. Study of implantation sites in animals allowed to survive 10-14 days before sacrifice showed that the tumor cells had disappeared in a mass of reactive tissue.

About every fifth tumor was implanted in normal as well as in irradiated animals. No tumor cells were found after 8 days in the untreated hosts.

Results. On the basis of microscopic examination of the samples taken at times of transplantation, the results of implanting 100 human cancers in X-irradiated animals fall into 3 equal groups (Table I): (1) Failures: In these none of the implanted cells were in active mitosis after 8 days in the heterologous hosts even though surviving cancer cells might be present. (2) Single generation success: Here proliferating cells were present at the time of the first transplantation attempt but none at the second. (3) Multiple generation success: Vigorous mitosis was present for two or more generations.

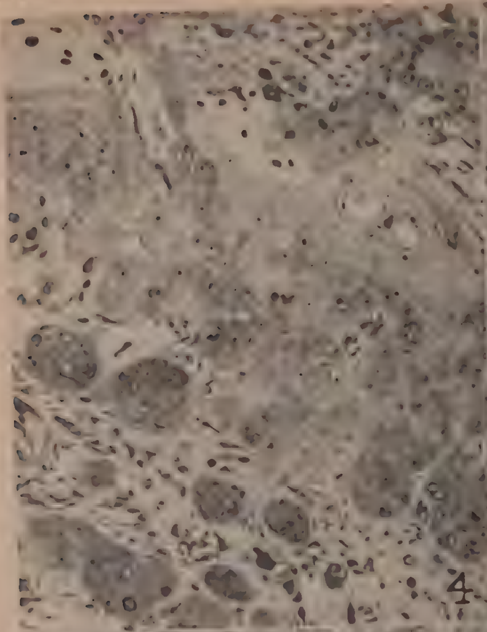
With very few exceptions the findings were the same for a particular tumor whether implanted in mice or rats, and, in general, they were similar for all the animals of a group. The size of the nodule at an injection site was no indication of the success or failure of the implantation since it might be composed entirely or in large part of reactive tissue.

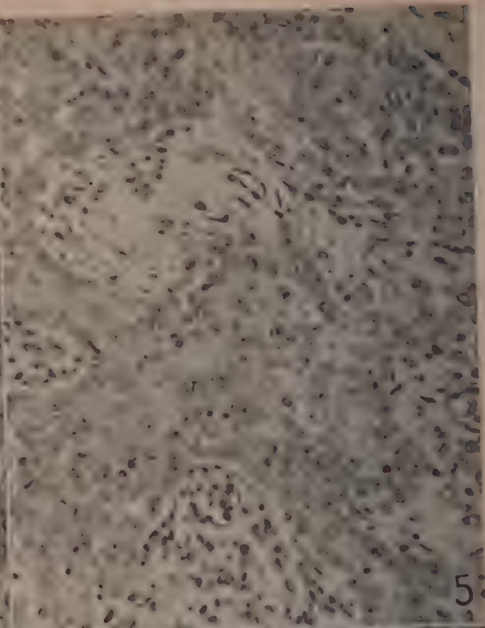
Epidermoid carcinomas grew most successfully and a number were transferred for several generations. This type of tumor was particularly satisfactory, furthermore, because it is easily distinguished from the subcutaneous, or reactive, tissues of the hosts (Fig. 1-6). Other types of neoplasms sometimes grew equally well (Fig. 7-9). The 10 infiltrating duct carcinomas of the breast, on the contrary, did poorly even though female hosts were used. This might have been due to the large amount of stroma and comparatively few cancer cells characteristic of this tumor.

The growths obtained from the sieved cell suspensions were as good as those from the minced tissue implantations though more cells survived when the minced material was used. Fig. 2 and 3 show a 3rd generation transplant 25 days after removal from the patient, and Fig. 7 is also a 3rd generation, 22 days after removal. The large masses of cancer cells, such as those in Fig. 2, necessarily indicate multiplication since the implanted sieved suspensions were composed of individual or at most, small clumps of cells.

It is of interest that almost all the tumors which survived for 2 generations or more in the heterologous host showed considerably more mitotic activity than did the original implants (Fig. 1-6), and none had less. It may be that some selective adaptation of cells occurred. Implants in successive generations were well vascularized (Fig. 6, 9), furthermore, whereas those that only survived for only one generation often were not. As shown in Table I, 6 tumors were transferred successfully for 4 generations and one was transplanted for 9. In many instances no attempt was made to transfer the material beyond the third generation since it was found that the tumors eventually died out. The reason appears to lie in the fact that only unusual mitotic activity during the short 8-day period before recovery of the animal from irradiation could make up for the considerable mechanical loss of cells at the time of transfer. The small groups of tumor cells still left in the later generations showed no diminution of vitality, however, for often fully a fifth of them were in mitosis.

Two factors were correlated with failure of the original implantation to survive: (1) Infection. Of 33 tumors in the group of failures, 13 were contaminated as compared with none in the more successful groups. (2) Delay in implantation. Time between removal of the growth from the patient and implantation in





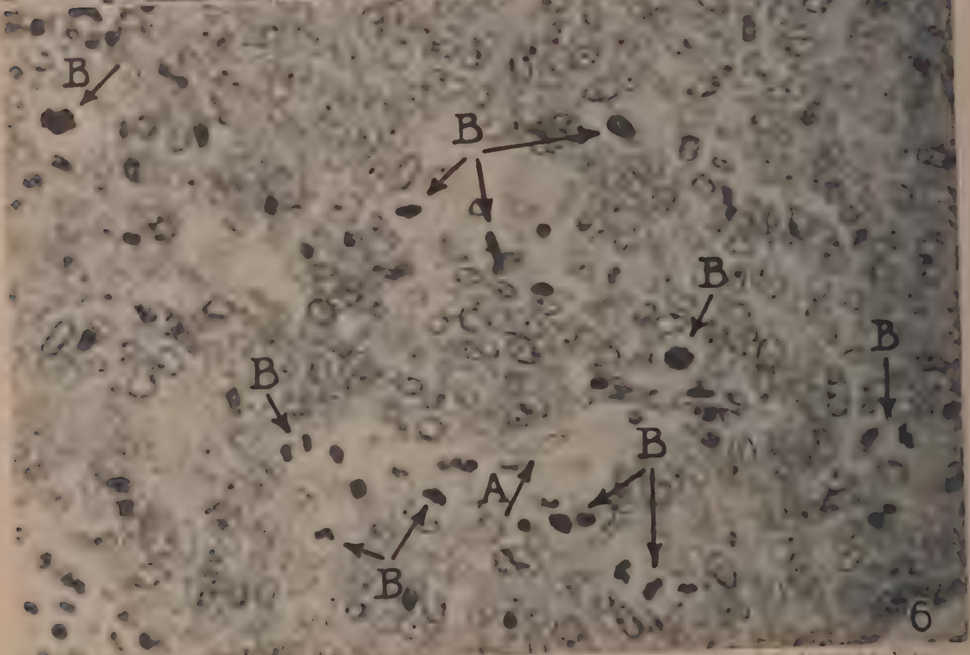
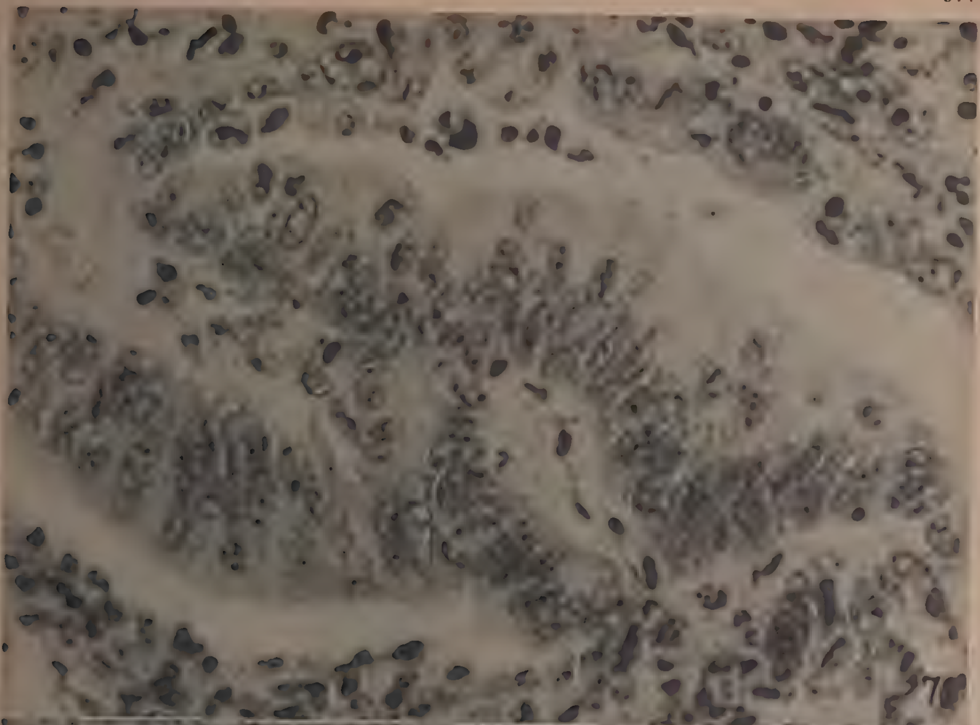


FIG. 4. Transplant of a recurrent squamous ca. after 2 generations (16 days) in X-irradiated rats. × 200.

FIG. 5. Transplant of a metastatic squamous ca. from radical neck dissection after 2 generations (16 days) in X-irradiated rats; tumor well vascularized and similar to original specimen. × 200.

FIG. 6. Transplant from epidermoid ca. of tongue after 2 generations (17 days) in X-irradiated rats; note blood vessel (arrow A) and numerous mitoses (arrows B). × 600.



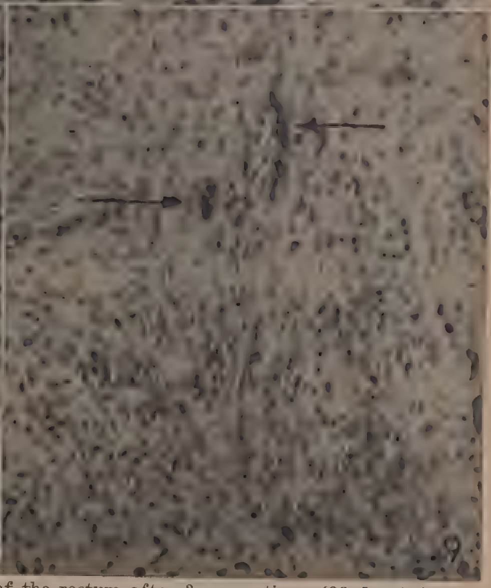


FIG. 7. Transplant of an adenocarcinoma of the rectum after 3 generations (22 days) in X-irradiated mice. The resemblance to the tissue of origin is striking. Original implantation a sieved suspension of cells. × 800.

FIG. 8. Fibrosarcoma of the leg, original specimen. × 180.

FIG. 9. Transplant of fibrosarcoma (Fig. 8) after 2 generations in X-irradiated rats. Growth very similar to original and well vascularized (arrows point to small blood vessels). × 180.

the experimental animals was found to be most important. As this exceeded one hour, less favorable results were obtained.

The ideal demonstration of viability would have been reimplantation of the material grown in heterologous hosts back into the original human donor. This was not feasible, and so tissue culture was employed to provide confirmatory data. Pieces of 3 original epidermoid carcinomas were cultured in roller tubes and the sheets of epidermoid cells which grew out were compared with those obtained in tissue culture from the subcutaneous nodules of the first, second, and, in one case, third animal transplantations of the same growths; they were quite similar. Some mouse or rat fibroblasts were present but the typical epithelial cells predominated. One of these epidermoid carcinomas was highly anaplastic with many multinucleated cells. This same type of cell grew out in the tissue cultures from the original tumor and from the second transplant nodule. Unfortunately, no effort was made to culture material from the first transplant generation.

Summary. (1) Of 100 human neoplasms implanted subcutaneously in X-irradiated rats and mice as sieved or minced cell suspensions, 33 had no mitotic cells at the end of the first generation (8 days), 34 showed cellular proliferation at the end of the first generation but could not be transferred successfully to a second, and 33 were transplanted for 2 generations or more with evidence of active growth and vascularization. (2) Epidermoid carcinomas were most successfully propagated. (3) Failures were in part due to infection and to delay between surgical removal of the tumor and its implantation in the heterologous hosts.

Received May 14, 1951. P.S.E.B.M., 1951, v77.

A Microbiological Method for the Determination of L- and D-Aspartic Acids.* (18855)

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It was shown previously(1) that D-aspartic acid supported only limited growth of *Leuconostoc mesenteroides* P-60 with relatively short incubation times in asparagine-free media. When low concentrations of L-asparagine were present, however, the growth response to D-aspartic acid was nearly the same as, or better than, that to L-aspartic acid. This suggested that *Leuconostoc mesenteroides* might prove useful for the determination of both aspartic acid enantiomorphs in hydrolysates of biological materials. The method which was subsequently developed for this purpose is described.

Experimental. The stock standard solution contained 4.00 mg of L-aspartic acid,‡ 1.00 mg of D-aspartic acid,‡ and 0.05 ml of 12 *N* hydrochloric acid per ml. It was stored at room temperature and diluted as required just before using. The diluted standard solutions were added in amounts of from 0.2 ml to 1.0 ml per tube to give a range of total aspartic acid concentrations of from 0 to 70 γ per tube in steps of 5 γ per tube.§ Each level was tested in triplicate. The standard was set

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[*] The author is indebted to Professor Marcel Florkin for sponsoring this work at the University of Liège, to Dr. H. Sarlet, Dr. Gh. Duchâteau, and Mlle L. Buyel for valuable assistance, and to Mme L. Pasleau for making the nitrogen determinations.

[†] U. S. Fulbright Scholar. Present address: Department of Chemistry, University of California, Los Angeles.

1. Camien, M. N., and Dunn, M. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 74.

[‡] These products, the same as those described previously(1), were kindly supplied by Dr. Max S. Dunn.

[§] A 3 *N* NaCl solution was added, taking into account amount of NaCl formed in neutralizing HCl acid present.

up in the same manner for both L- and total aspartic acid assays, but since only 80% of the aspartic acid in the standard solutions was L-aspartic acid, the L-aspartic acid standard curve ranged from 0 to 56 γ per tube in steps of 4 γ per tube.

Samples. Dried cells of *Lactobacillus arabinosus* 17-5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60 were prepared according to the procedure employed previously(2). 1.5 g of each of these preparations and of a sample of casein ("vitamin test casein," General Biochemicals, Inc.) were hydrolysed by refluxing for 20 hours with 15 ml of 8 N hydrochloric acid. Each hydrolysate was filtered into a 100 ml volumetric flask, diluted to volume, and stored at room temperature. Aliquots were diluted further as required on the day of each experiment. Five levels of diluted hydrolysate, each in triplicate and ranging from 0.2 ml to 1.0 ml per tube, were tested for each sample.

Bromthymol blue was added during the preparation of diluted standard and sample solutions to facilitate neutralizing, and each final solution was prepared to contain 17.5 mg of sodium chloride[¶] and 0.008 mg of brom thymol blue[‖] per ml at about pH 6.2. A "blank" solution containing the same concentrations of sodium chloride and bromthymol blue was employed to bring the volume in each test tube to 1.0 ml before adding the medium.

The basal medium was the same as that employed previously(1), except that sodium chloride, norleucine, and norvaline were omitted and the concentration of each amino acid was increased by 50%. The basal medium was modified for the determination of L-aspartic acid by adding 60 mg of D-aspartic acid per 200 ml of medium (sufficient for 100 assay tubes, each containing 3 ml final volume after adding medium and sample) and adjusting the pH to 5.5. The basal medium was modified for the determination of total (L- plus D-) aspartic acid by adding 4.5 mg of L-asparagine monohydrate per 200 ml of medium and adjusting the pH to 6.5. 2 ml of medium were used per tube. The racks of tubes were covered with clean towelling, heated rapidly to 120° in the autoclave, held at that temperature for 10 minutes and cooled rapidly before inoculating.

The inoculum of *Leuconostoc mesenteroides* P-60 was the same as that employed previously(1), except that the growth from 7 ml of inoculum medium was resuspended in 100 ml of sterile saline, and 0.1 ml of this suspension was used to inoculate each tube. The inoculated tubes were incubated at 35°. The incubation period was from 39 to 42 hours for the L-aspartic acid determinations and from 63 to 66 hours for the determinations of total aspartic acid. The tubes were heated in the autoclave at 100° for 20 minutes to terminate growth. Titrations and calculations were carried out as described previously(3).

Discussion. Rather large changes in the "blank" response of *Leuconostoc mesenteroides* to L-aspartic acid were found to accompany variations in incubation time and temperature when the basal medium contained an excess of D-aspartic acid (Fig. 1). It was particularly desirable with the L-aspartic acid determinations, therefore, to be certain that the incubation temperature was precisely the same for each of the assay tubes and to terminate growth simultaneously in all the tubes at the end of a given experiment. The incubator used in the present experiments was found to produce a highly uniform temperature, although it seems likely that the reliability of a constant temperature water bath might be much superior in this respect. The incubation period for the L-aspartic acid determinations was kept relatively short (39 to 42 hours at 35°) to avoid excessively high "blank" titrations.

The initial pH of the medium was also found to influence the "blank" response to L-aspartic acid (Fig. 2). This effect did not

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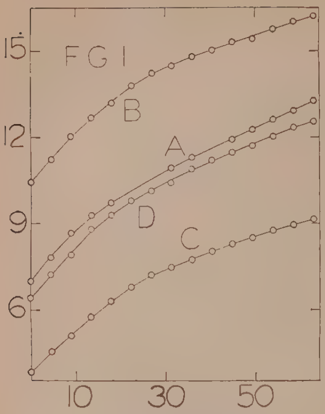
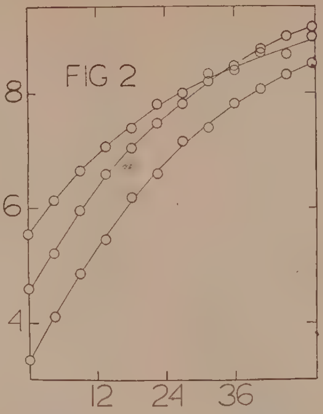
2. Camien, M. N., Salle, A. J., and Dunn, M. S., *Arch. Biochem.*, 1945, v8, 67.

[¶] 1 ml of a 0.8 mg per ml solution of bromthymol blue in 5% ethanol was added per 100 ml of final dilution.

[‖] The method of setting up the standard was analogous to that described previously(3).

3. Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., and Reiner, P. J., *Univ. Calif. Publ. Physiol.*, 1949, v8, 293.





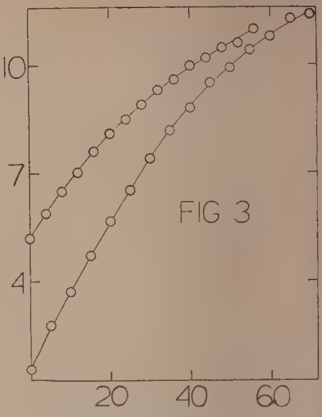


FIG. 1. Response to L-aspartic acid in medium containing 20 mg % D-aspartic acid. Curves A and B were obtained with 38 and 62 hr incubation, respectively, at 39°. Curves C and D were obtained with 38 and 62 hr incubation, respectively, at 33°. The values on the horizontal scale represent the γ of aspartic acid per tube, and values on vertical scale are calculated as ml of 0.01 N NaOH required to titrate 1 ml of culture.

FIG. 2. Response to L-aspartic acid with 38 hr incubation in media containing 20 mg % D-aspartic acid. Initial pH of medium was 5.5, 6.0, and 7.0, respectively, for the lower, middle, and upper curves. Coordinates are as in Fig. 1.

FIG. 3. Typical standard curves for determination of L-aspartic acid (upper curve) and total (L- plus D-) aspartic acid (lower curve). Coordinates are as in Fig. 1.

appear to result from destruction of nutrients in the medium during sterilization at the lower pH values since essentially the same results were obtained whether the pH was reduced before or after sterilization. An initial pH of 5.5 in the L-aspartic acid assay medium appeared to be best, and although the "blank" was relatively high even under these conditions (Fig. 3), the assays by this method were reproducible and accurate to a satisfactory degree (Tables I and II).

When the basal medium contained low concentrations of L-asparagine, mixtures of L- and D-aspartic acids were usually slightly more active than L-aspartic acid alone. For this reason a mixture of L- and D-aspartic acids was used rather than L-aspartic acid alone as standard in the determinations of total aspartic acid. By using a 4 to 1 ratio of L-aspartic acid to D-aspartic acid it was possible to use the same standard solutions for the total aspartic acid standard (range, 0 to 70 γ per tube) as for the L-aspartic acid standard (range, 0 to 56 γ per tube). Typical standard curves for L-aspartic acid and total aspartic acid determinations are shown in Fig. 3.

Recoveries of L-aspartic acid from samples containing no D-aspartic acid were low (because L-aspartic acid is less active than mixtures of L- and D-aspartic acids under these conditions) as determined by the total aspartic acid assay method (Table I). With this exception, however, satisfactory recoveries of L- and D-aspartic acids from mixtures of pure amino acids (Table I), from casein hydrolysate (Table I), and from hydrolysates of bacterial cells (Table II) were consistently obtained.

The aspartic acid values found at the different test levels were found to be reproducible to a satisfactory degree with both the method for L-aspartic acid and that for total aspartic acid. The mean per cent deviation from the mean was calculated for each "final" value (the mean of the individual values found at the 4** or 5 sample levels), and the average mean deviation from the mean (calculated from all the values obtained by these methods) was 2.1% with the method for L-

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** Five sample levels were tested in each case, but when one of the 5 values was different from the value nearest it by an amount greater than the range of the other four values, this value was omitted in calculating the mean value.

TABLE I. Recovery of L- and D-aspartic Acids from Mixtures of Pure Amino Acids and from Casein Hydrolysate.

Added		Found					
		L-aspartic acid			Total aspartic acid		
L-aspartic acid	D-aspartic acid	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
		% in amino acid mixture*					
100		99.6	99.6	99.6	90.4	94.6	93.6
50	50	49.5	49.2	49.2	97.6	98.4	99.4
0	100	0	0	0	98.6	98.8	100.2
3.45	0	3.54	3.41	3.69	3.21	3.25	3.21
2.76	.69	2.98			3.48		
2.07	1.38	2.12			3.44		
1.73	1.72	1.71	1.74	1.81	3.45	3.40	3.48
.69	2.76	.71			3.40		
0	3.45	0	0	0	3.38	3.43	3.47
		g per 100 g of casein†					
0	0	7.61	7.54	7.61	7.88	7.95	7.71
8.10	0	15.41	15.73	15.31	15.48	15.89	15.60
0	8.10	7.44	6.99	7.22	15.79	15.87	15.89

* Mixtures containing 3.45% (total) aspartic acid contained also 6.9% each of DL-alanine, L-arginine • HCl, L-cystine, L-glutamic acid, L-histidine • HCl • H_2O, DL-isoleucine, L-leucine, DL-methionine, DL phenylalanine, L-proline, DL-serine, L-tryptophan, L-tyrosine, and DL-valine.

† N was determined in the hydrolysate and the casein concentration calculated as N × 6.25. The hydrolysate contained 13.17 g of soluble N per 100 g (uncorrected) of casein.

TABLE II. Recovery of L- and D-aspartic Acids from Hydrolysates of Bacteria.

	g per 100 g bacterial protein*			
	Added		Found†	
Bacteria	L-aspartic acid	D-aspartic acid	L-aspartic acid	Total aspartic acid
L. arabinosus	0	0	8.71 (8.49–9.17)	9.18 (9.07–9.33)
	9.15	0	18.58	17.92
	0	9.15	8.74	18.14
S. faecalis	0	0	7.04 (6.84–7.26)	9.19 (9.09–9.34)
	9.26	0	16.37	18.25
	0	9.26	7.03	18.13
L. mesenteroides	0	0	7.74 (7.64–7.89)	11.10 (10.83–11.34)
	9.4	0	17.07	20.40
	0	9.4	7.40	20.28

* N was determined in the hydrolysates and the protein calculated as N × 6.25. The hydrolysates of *L. arabinosus, S. faecalis* and *L. mesenteroides* contained, respectively, 9.72, 9.47 and 9.61 g of soluble N per 100 g of bacteria (dry wt, uncorrected for any residual moisture which might have been present).

† The values given for bacteria without additions of aspartic acid are averages of four independent determinations, ranges being given in parentheses. Other values represent single determinations.

aspartic acid and 1.3% with that for total aspartic acid.

Only small amounts of D-aspartic acid (the difference between L-aspartic acid and total aspartic acid) were detected in the hydrolysates of casein and *Lactobacillus arabinosus* cells (Tables I and II). Much more D-aspartic acid was found in the hydrolysates of *Streptococcus faecalis* and *Leuconostoc mesenteroides* cells (Table II), however, amounting to 23.4% and 30.3%, respectively, of the total aspartic acid. Although it is not impossible that such large amounts of D-aspartic acid may have been produced by racemization, it seems likely that the values found represent the approximate amounts of D-aspartic acid originally present in the protoplasm of these bacteria. That a relatively large amount of D-aspartic acid was found in the cells of *Leuconostoc mesenteroides* (Table II) lends support to the suggestion made previously(1) that D-aspartic acid may be an essential metabolite for this organism.

The average value, 7.85%, found for total aspartic acid in casein (Table I) was considerably greater than that reported by Hac and Snell(4). However, the latter was corrected only for the moisture content of the casein sample, and the true value must have been somewhat higher, if impurities other than moisture were present in significant amounts. The values obtained in the present experiments were calculated on the basis of 16% nitrogen in casein, and the nitrogen was determined in the hydrolysate. This procedure would give high values either if the true value for nitrogen in casein were significantly less than 16% or if a significant amount of the casein nitrogen were insoluble in the hydrolysate (insoluble humin was removed by filtration before the nitrogen determination was made). The use of a more painstaking procedure did not seem warranted in the present experiments because the available casein sample was not one which had been carefully prepared and highly purified.

Summary. A microbiological procedure was developed for the determination of L- and D-aspartic acids with *Leuconostoc mesenteroides* P-60. Added L- and D-aspartic acids were recovered adequately from amino acid mixtures and hydrolysates of casein and bacterial cells. Only small amounts of D-aspartic acid were detected in casein and in *Lactobacillus arabinosus* cells, but in *Streptococcus faecalis* and *Leuconostoc mesenteroides* cells about one-fourth to one-third of the total aspartic acid was of the D- configuration.

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Received May 17, 1951. P.S.E.B.M., 1951, v77.

A Modified Procedure for Determining Inulin Space.* (18856)

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Although the inulin space may be a somewhat vague entity, inulin has recently come into importance as a tool in the estimation of extracellular space. The inulin space is determined by dividing the total amount of inulin present in the animal by the plasma concentration after an equilibrium has been established between the plasma and the interstitial compartments. The simplest way to compute this quantity of retained inulin is to subtract the amount excreted up to the time of equilibration from the total amount given. Such a procedure has been criticized by Kruhøffer(1) on the grounds that the subtrahend is a relatively large fraction of the minuend and a small per cent error in either of these factors is magnified considerably in calculating the difference. Gaudino and Levitt(2) claimed that following a 2-hour inulin infusion, all the inulin is excreted within 4-6 hours. Therefore, they tried to obviate the difficulty inherent in the original method by computing inulin space as the ratio of the quantity of inulin excreted within 5 hours following termination of the infusion to the concentration of inulin in the plasma.

In using their method it was found that a much longer time was required to approach complete recovery. Therefore we have attempted to improve the accuracy and expediency of the inulin space determination by introducing a slight modification of the original method.

Methods. Thirteen experiments were performed on 10 female dogs under pentobarbital anesthesia. Pure dry inulin which was fructose and pyrogen free† was accurately weighed

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* Supported by a grant from the U. S. Public Health Service.

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† Pfanstiehl inulin which was checked repeatedly against recrystallized standards from different laboratories.

TABLE I. Data from 6 Inulin Experiments in Which Post-Infusion Urine Collection Was Limited to 5 Hr.

Dog	Inulin administered, mg	Inulin excreted during infusion, % of admin.	Inulin excreted in 5 hr after infusion, % of admin.	Inulin unrecovered in 5 hr % of admin.	Inulin unrecovered in 5 hr % of remain.*
1	1932	59.5	35.2	5.3	13.2
2	1757	65	28.9	6.1	17.3
2	2022	70.6	27.3	2.1	7.3
3	2296	61.8	37	1.2	3.2
3	2417	58.1	34.8	7.1	16.9
3	2010	67.4	27.9	4.7	14.4
Avg		63.7	31.9	4.4	12.1

* Amt remaining in dog at moment infusion is terminated.

and dissolved in a known volume of warm saline. After plasma and urine blanks had been obtained this warm inulin solution was administered intravenously at a constant rate with a mercury pump over a 2-hour period. This time interval was found by Gaudino and Levitt to be adequate to attain a uniform concentration of inulin throughout the extracellular space. However, this may not be sufficient time in all cases since Leonards(3) found that 6 hours were required to reach equilibrium in 2 nephrectomized dogs following a single injection of inulin. Urine was collected during this equilibration period. In order to be certain that none of the inulin in solution had precipitated out during the infusion period the concentration of inulin in the few remaining cubic centimeters was determined by chemical analysis. This checked with the actual value within the range of error of the experimental method. Precisely at the termination of the infusion plasma samples were drawn, the bladder was washed, and further urine collections were made. The amount of inulin remaining in the infusion flask and tubing at the end of 2 hours was then measured to within 0.1 to 0.2 cc. Inulin space was calculated by dividing the difference between the amount of inulin administered and the amount excreted during the infusion, by the plasma concentration, as well as by the method of Gaudino and Levitt. In the first 6 experiments urine was collected for 5 hours after the infusion was stopped, while in the last seven experiments collection was continued for from 8-11.5 hours. All inulin determinations were made by the method of Higashi and Peters(4). As a check on the method, known amounts of dry inulin were added to urine or saline and recovery studies were carried out with each experiment. The mean of the per cent difference between the known concentration of inulin and that determined chemically was 1.81 with a S.D. of ±1.24. Correction for dead space was not made since it has been amply shown that the capacity of the ureters and pelves vary from time to time and that appearance time does not bear a linear relationship to the rate of urine flow(5,6). Besides, this correction is only of importance in calculating the inulin space and has no bearing on the inulin recovery data.

Results. Table I lists the data compiled from the first 6 experiments. On the average 63.7% of all the inulin given was excreted during the 2 hour equilibration period, while an additional 31.9% was excreted in the following 5 hours. In none of these experiments was all the inulin recovered in 5 hours. An average of 4.4% of the total amount of inulin administered remained in the dogs at the end of 5 hours.

Expressed in per cent of the amount of inulin present in the dog at the moment the infusion was interrupted, an average value of 12.1% was obtained. Since this quantity of inulin is the numerator in the calculation

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TABLE II. Data from 7 Inulin Experiments in Which Post-Infusion Urine Collection Was Extended Beyond 5 Hr.

				Inulin unrecovered in 5 hr			Inulin excreted after 5 hr		Unrecovered inulin	
Dog	Inulin admin., mg	Inulin excreted during infusion, % of admin.	Inulin excreted in 5 hr after infusion, % of admin.	% of admin.	% of remain.*	Collection time beyond 5 hr, hr	% of admin.	% of remain.*	% of admin.	% of remain.*
4	1686	73.2	22.3	4.5	17	5	2.8	10.4	1.7	6.6
5	1862	59.9	30.3	9.8	24.4	5.1	3.1	7.8	6.7	16.6
6	1707	70.7	25.7	3.6	12.2	6.5	.3	1	3.3	11.2
7	2163	73.7	21.4	4.9	18.8	6.3	1.4	5.4	3.5	13.4
8	1914	72	26.5	1.5	5.2	3.3	1.7	6	–.2	–.8
9	2290	58.8	32.4	8.8	21.5	3	4.1	9.9	4.7	11.6
10	2079	46.4	39.5	14.1	26.3	3.2	4.7	8.8	9.4	17.5
Avg		65	28.3	6.7	17.9	4.6	2.6	7	4.1	10.9

* Amt remaining in dog at moment infusion is terminated.

of the inulin space, an equivalent error is incurred in this determination.

In Table II data are recorded from a second group of experiments in which urine was collected for longer than 5 hours. In this series too, inulin recovery was incomplete after 5 hours. The average amount missing is greater than in the first 5 experiments, while the amount of inulin recovered after 5 hours was 7% of that present in the dog at the time of equilibration.

In one experiment (dog 8) inulin recovery was complete in 8.3 hours while in the other 6, significant quantities of inulin remained in the dog after 8.0 to 11.5 hours of urine collection. Inulin space determinations, using the inulin recovered in 8.0 to 11.5 hours after the infusion, show an average error of 10.9%.

These data show complete inulin recovery was not attained in any experiment in 5 hours and in only one out of 7 experiments in 8 to 11 hours, which is in keeping with the fact that inulin is excreted exponentially, approaching complete elimination asymptotically(7,8).

Discussion. There are two distinct advantages in calculating the inulin space as we have done. First of all the error is smaller. When the inulin space is computed using the amount of inulin collected in 5 hours as representing the total amount remaining at the time of equilibration, an error of 3.2 to 26.3 averaging 15% was obtained in 13 experiments. To this must be added the error of the method for inulin determination in urine. Extension of the urine collection period to 8 to 11 hours reduces the average error to 10.9%. However, by accurately weighing the dry inulin and dissolving it in a known quantity of saline, the amount administered can be determined within a 0.1% error. Using the method of Higashi and Peters(4) the average error in determining the inulin concentration in the urine excreted during the infusion was found to be 1.81%. Since two-thirds of the administered inulin is excreted during the infusion, a total error of 3.8% is obtained in the calculation of the unexcreted inulin and consequently an equal error in the estimation of the inulin space. Therefore the failure of the dog to eliminate all of the inulin in 5 hours of urine collection introduces an error about 3 times as great as that obtained by calculating this figure from the difference of the amount infused and the amount excreted during the infusion. Extension of the urine collection period only partially reduces the error in the method of Gaudino and Levitt, and necessitates a long drawn out experiment of over 11 hours.

Secondly, the method of differences which we have employed is less time consuming since the entire procedure is limited to the period

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necessary for equilibration to be reached. With increased extracellular volume the time for equilibration to be reached is longer and the time for post-infusion urine collection becomes extremely long, making the inulin space determinations impractical under such conditions.

No satisfactory explanation can be offered to account for the difference in inulin recovery in our hands and those of Gaudino and Levitt. The possibility that light anesthesia significantly altered the rate of diffusion of inulin in our experiments has not been excluded.

Summary. 1. Inulin recovery was found to be incomplete in five hours after the termination of a two-hour inulin infusion. Since the unrecovered inulin amounted to an average of 15% of the inulin present in the dog at the time of equilibration, an equivalent error was introduced into the calculation of the inulin space by the method of Gaudino and Levitt. 2. By administering a solution made from pure, accurately weighed, dry inulin, and determining the inulin content of the urine excreted during the infusion, the amount of inulin remaining in the dog can be computed with an average error of only 3.8%. In addition, the time needed for urine collection is greatly reduced.

Received May 13, 1951. P.S.E.B.M., 1951, v77

Role of Fat on Utilization of Lactose in Milk by Rats and Rabbits. (18857)

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The role of fat on the utilization of lactose or galactose by rats was reported by several workers(1-5). Rats cease to grow and die if lactose or galactose is the only source of carbohydrate. Growth is continued and death is averted if fat is included in the diet(1). Rats fed skim milk excrete galactose in urine and addition of fat prevents this loss in urine (2). The percentage of ingested galactose excreted in the urine is inversely proportional to the percentage of fat in the diet(4). It is, however, not clear how fat helps in the utilization of galactose. In a normal animal ingested galactose is converted into glycogen in the liver(6). It is probable that in the absence of fat in the diet liver can not convert galactose into glycogen. Glycogen content of the liver of skim milk-fed and whole milk-fed rats was, therefore, determined. As galactose is mainly present in the nervous tissue as a constituent of cerebroside it was of interest to study whether skim milk-fed rats could utilize galactose for the synthesis of cerebroside of the brain. To study if fat is necessary for the metabolism of galactose in other species of animals the effect of feeding skim milk on urinary excretion of galactose by rabbits was also studied.

Experimental. Effect of feeding skim milk and whole milk on urinary excretion of galactose and on glycogen deposition in the liver of rats. Male rats weighing between 68 and 98 g were taken. Eight rats were fed *ad libitum* mineralised(2) skim milk for 15 days. Each rat was kept in a metabolism cage and the urine was collected under toluene. For the next 2 days the rats were fed daily 80 cc of skim milk per rat. Nine rats were fed mineralised whole milk for 15 days and for the next 2 days each of the rats received daily 40 cc of whole milk isocaloric with 80 cc of skim milk. The rats also received 2 drops of a concentrate of vit. A, D and K twice a

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TABLE I. Urinary Excretion of Galactose and Glycogen Value of Liver of Rats Fed Whole Milk or Skim Milk for 15 Days. Whole milk-fed rats received isocaloric amount of milk as consumed by skim milk-fed rats for last 2 days of experiment.

	No. of rats used	Wt at death, g	Ingested galactose excreted in urine, %	Glycogen per 100 g liver, mg
Skim milk-fed rats	8	89 (82–100)	22.5 (16.2–32.4)	12.6 (9–17)
Whole milk-fed rats	9	98 (84–120)	0	776 (405–1847)
Diff. of means				763.4
Std error of diff.				161
t				4.7*

* Highly significant.

week. Sugar excreted in the urine was estimated with Benedict's quantitative reagent and calculated as galactose after standardizing the reagent against pure galactose. The sugar in the urine was identified as galactose(2). On the eighteenth day of experiment the rats were stunned by a blow on the head, neck veins were cut and livers were removed. Glycogen in the liver was estimated by the method of Grattan and Jensen(7). Percentage of the ingested galactose excreted in the urine on the last day of experiment and the glycogen value of the liver are given in Table I.

Effect of feeding skim milk and whole milk on cerebroside content of the brain and fat content of liver of rats. Weanling rats weighing between 31 and 56 g were divided into 2 groups. One group was fed *ad libitum* for 7 weeks skim milk prepared by mixing 10 g skim milk powder with 90 cc water in a Waring blender. The milk was mineralised(2). The second group of rats was fed for 7 weeks whole milk prepared by mixing 12 g of whole milk powder with 88 cc water. The rats also received 2 drops of a concentrate of vit. A, D and K twice a week. Rats were weighed on alternate days and galactose in urine was estimated daily. Skim milk-fed rats excreted galactose at the later stage of the experiment. The whole milk-fed rats did not excrete any galactose. The animals were killed after 7 weeks and brain and livers were removed for the estimation of cerebroside and fat respectively.

Estimation of cerebroside. The brain was weighed and spread on a washed filter paper of 9 cm diameter. The filter paper was rolled and placed inside a glass thimble with perforated walls, which was suspended from the lower end of a condenser fitted into a 125 cc conical flask with a ground glass joint. The brain was defatted by extraction with acetone in a boiling water bath for 4 hours and then extracted with aldehyde-free ethanol for another 4 hours. The ethanol extract was transferred into a 50 cc centrifuge tube and kept in a refrigerator overnight. The precipitate was discarded and the supernatant was made up to a definite volume with absolute ethanol. Cerebroside was determined in an aliquot of the extract by the method of Brand and Spery(8) slightly modified. The results are given in Table II. *Estimation of liver fat.* Fat content of livers of rats was estimated according to the method described by Chattopadhyay, Nandi and Banerjee(9). The results are given in Table II.

Relation of fat to utilization of lactose by the rabbit. Adult rabbits did not consume milk when supplied exclusive of other diets and died of inanition. Rabbits given milk from birth learn to drink it and gain in weight. Six rabbits born in this laboratory were given milk from birth, 3 received skim milk and the rest whole milk for 2 months. The rabbits were housed in individual metabolism cages and urine was collected under toluene. Sugar in the urine was estimated daily. None of the

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TABLE II. Cerebroside Content of Brain and Fat Content of Liver of Rats Fed Whole Milk or Skim Milk *ad libitum* for 7 Weeks.

	No. of rats	Wt at death, g	Gain in wt per week, g	Cerebroside per 100 g brain, mg	Fat per 100 g liver, g
Skim milk-fed rats	9	96 (80–106)	8 (6–10)	741 (412–982)	5 (4.2–6)
Whole milk-fed rats	8	132 (111–161)	12 (8–16)	676 (502–876)	6.8 (6.1–7.6)
Diff. of means			4	65	1.8
Std error of diff.			1.18	83	.35
t			3.4	.8*	5.2

* Except for this value, all values of *t* are highly significant.

rabbits excreted sugar in the urine.

Results. Skim milk-fed rats excreted galactose in urine. The glycogen values of their livers were significantly lower than the values obtained in rats fed isocaloric amounts of whole milk. Considerable amount of fat was present in the liver of skim milk-fed rats. The cerebrosides content of brains of whole milk and skim milk-fed rats did not differ significantly. Skim milk-fed rats grew at a lesser rate than the whole milk-fed rats. Rabbits fed skim milk for 2 months did not excrete sugar in the urine.

Discussion. The diminished glycogen values of the liver of skim milk-fed rats possibly indicate that galactose is not converted into glycogen in the liver of rats in the absence of fat in the diet. The galactose, therefore, goes into the blood stream and being a low threshold substance is excreted by the kidney. Galactose, however, can be partly utilized in the absence of liver(10). The normal cerebrosides content of skim milk-fed rats indicate that galactose is partly utilized by these rats in the formation of cerebrosides. The presence of considerable amounts of fat in the liver of skim milk-fed rats might be due to partial conversion of galactose into liver fat. Fat is not necessary for the utilization of lactose by the rabbits.

Summary. (1) The glycogen and fat contents of the liver are significantly diminished in the rats fed skim milk. (2) The cerebroside content of the brain of the skim milk-fed and whole milk-fed rats did not differ significantly. (3) Urinary excretion of galactose by the skim milk-fed rats might be due to non-conversion of galactose into glycogen in the liver. (4) The skim milk-fed rats utilized galactose to form cerebrosides of the brain and fat of the liver. (5) Unlike rats, rabbits fed skim milk for 2 months did not excrete galactose in urine.

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Received May 21, 1951. P.S.E.B.M., 1951, v77.

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